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The Effects of Polyunsaturated Fatty Acids on Immune function in Obese Insulin Resistant Rodents

by

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ABSTRACT

Altered immune responses have been reported in obese individuals, although the exact impairments have not been established. Conjugated linoleic acid (CLA), a group of positional and geometric isomers of linoleic acid, has been reported to beneficially alter immune function in healthy and inflammatory states. Long chain (n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have established anti-inflammatory and immunoregulatory properties in autoimmune inflammatory states. However, the effects of these PUFA on immunity in obesity are unknown. Therefore, the objective of this thesis was to determine the effects of obesity and dietary PUFA on immunity.

The *fa/fa* Zucker rat had lower Concanavalin A (ConA)-stimulated IL-2 production (impaired T-cell function) and greater mitogen-stimulated IL-1β, TNF-α and IL-6 (inflammatory) cytokine production compared to lean rats ($P<0.05$). Feeding the *cis*9,*trans*11 or *trans*10,*cis*12 CLA isomer singly or in combination to *fa/fa* Zucker rats resulted in incorporation into splenocyte phospholipids, but to a lesser extent than lean rodents. Feeding *cis*9,*trans*11 CLA to *fa/fa* rats improved IL-2 and IL-10 production to levels similar to lean rats fed the same diet. Obese rats fed *trans*10,*cis*12 CLA had lower LPS-stimulated IL-1β and TNF-α.

In a short-term study, there was no difference in T-cell stimulated IL-2 production and there was lower stimulated IL-1β and IFN-γ (inflammatory cytokine) production by obese JCR:LA-*cp* rats compared to lean rats ($P<0.05$). However, in a long-term intervention, production of IL-1β and the Th1 response (IL-2 and IFN-γ) were higher in obese rats compared to lean rats. Higher levels of protein kinase C-θ (PKC-θ) may partly

explain the higher IL-2 concentrations in obese rats. Obese rats fed fish oil (FO) had more long chain (n-3) PUFA and lower (n-6):(n-3) PUFA ratio in splenocyte phospholipids and lipid rafts. In the short-term intervention, obese FO-fed rats produced less IL-1β and IFN-γ without affecting IL-2 production. Whereas, high FO fed rats in the long-term intervention produced more IL-2, but this was not attributable to PKC-θ.

Overall, T-cell and inflammatory cytokine responses are impaired in rodent models of obesity. Careful consideration of the immune parameter of interest is required to determine which model is most suitable. Dietary EPA and DHA and CLA have beneficial effects on T-cell function and inflammatory cytokine production.

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List of Abbreviations

AA – arachidonic acid

ALA – alpha-linolenic acid

ANOVA – analysis of variance

APC – antigen presenting cell

BCA – bicinchoninic acid

BMI – body mass index

BSA – bovine serum albumin

CD – cluster of differentiation

CLA – conjugated linoleic acid

ConA – Concanavalin A

CRP – C-reactive protein

Ctl – control

CVD – cardiovascular disease

DC – dendritic cell

DHA – docosahexaenoic acid

DIO – diet-induced obesity

DPA – docosapentaenoic acid

DTH – delayed type hypersensitivity

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbant assay

EPA – eicosapentaenoic acid

FCS – fetal calf serum

FITC - fluorescein isothiocyanate

FO – fish oil

GALT – gut-associated lymphoid tissue

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HF – high fat

HFF – high fat fed

IFN– interferon

Ig - immunoglobulin

IL – interleukin

LA – linoleic acid

LPS - lipopolysaccharide

LSM – least square means

MCP – monocytes chemoattractant protein

MHC – major histocompatibility complex

MLN – mesenteric lymph node

MUFA – monounsaturated fatty acids

NFAT - nuclear factor of activated T-cells

NF-κB – nuclear factor kappa B

NK – natural killer

NO – nitric oxide

OVA - ovalbumin

PBS – phosphate buffered saline

PC – phosphatidylcholine

PE - phosphatidylethanolamine

R-PE - R-phycoerythrin

PHA - phytohemaglutinen

PKC-θ – protein kinase C-theta

PL - phospholipid

PPAR – peroxisome proliferator-activated receptor

PUFA – polyunsaturated fatty acids

PWM – pokeweed mitogen

SE – standard error of the mean

SFA – saturated fatty acids

TBS – tris-buffered saline

TBST – tris-buffered saline plus Tween 20

TCR – T-cell receptor

TFR – transferring receptor

Th – T-helper cell

TLR – toll-like receptor

TNF – tumour necrosis factor

UNS – unstimulated

1 Introduction and Literature Review

1.1 Obesity

Obesity, defined by a body mass index exceeding 29.9 kg/m^2, is one of the leading health concerns worldwide. Globally, it was estimated that 1.3 billion adults were overweight (BMI >25 kg/m^2, but ≤29.9 kg/m^2) or obese in 2005 and it was projected that these numbers will only continue to escalate (2). The most recent estimates for Canada reported in the Canadian Community Health Survey are that 36% of adults are overweight and 23% are obese (3). Obesity places an enormous burden on healthcare systems in both direct and indirect costs. Though often considered a condition of the Western world, increasing incidence of obesity has been observed around the world, including developing countries (5).

Obesity is linked with numerous health complications that affect both the quality and duration of life (7). Excessive body mass is associated with an increased risk of type 2 diabetes, hypertension, cardiovascular disease (CVD), certain forms of cancer, asthma, osteo-arthritis and depression (8). It is hypothesized that inflammation is the common pathological link among obesity, cardiovascular disease (CVD), type 2 diabetes and some types of cancer (9). Although the initiating factor for this low grade chronic inflammatory state is unknown, a few hypotheses exist. Both abnormalities in immune function and mediators secreted by adipose tissue have been implicated as causative factors (12).

1.2 The Immune System

Our immune system protects us from the invasion of foreign pathogens and tumours and facilitates recovery from tissue injury and infection. This complex and multifaceted system must also ensure that the host tissue is distinguished from foreign particles or altered self cells. Thus, immune cells must strike a precise balance between attacking the foreign object and protecting the host from excessive damage. Often this balance is achieved; however, there are certain conditions when the immune system becomes skewed, causing damage to the host. Unbalanced or inappropriate immune responses are responsible for allergic reactions, graft rejection, autoimmune disease and other chronic inflammatory conditions.

The immune system is comprised of two distinct, yet intertwined arms, the innate and acquired. The innate immune system is our first line of defense against foreign

pathogens and tissue injury and provides an immediate, but non-specific reaction. The acute inflammatory response is an integral component of this system and is classically identified by heat, redness, pain and swelling. Several immune cells function to eliminate the immune challenge and restore homeostasis, including neutrophils, natural killer (NK) cells, macrophages/monocytes and dendritic cells, and the granulocytes, mast cells, eosinophils and basophils.

Acquired immunity specifically targets foreign pathogens/antigens or altered self cells that have been presented by antigen presenting cells (APCs) expressing major histocompatibility complex (MHC) Class I and II molecules. The cells of this highly developed system include T and B-lymphocytes and are also involved in regulating inflammation and healing. This branch develops over one's lifetime in response to antigen or pathogen exposure.

1.2.1 T-cells

T-cells, which develop in the thymus, account for the greatest number of circulating lymphocytes and comprise the majority of cells in certain lymph nodes, the spleen and aspects of the gut-associated lymphoid tissue, including mesenteric lymph nodes (13). Activation of T-cells requires recognition of antigen/MHC complex via the T-cell receptor (TCR) (13). In addition, appropriate co-stimulation of CD28 by the APC, mainly via CD80 or CD86, is also necessary for T-cell activation (13).

T-cells are classified as T-helper (Th, CD4+) cells or cytotoxic T-cells (CTL, CD8+). CTL recognize antigen presented on MHC class I molecules, expressed on all cell types; whereas, Th cells recognize MHC class II molecules expressed on APCs (13). Th cells are further classified as Th1 or Th2 depending on the pattern of cytokines secreted. The type of effector Th cell (i.e. Th1 or Th2) that develops depends on several factors, including the type of antigen presented, cytokine environment and the co-stimulatory signals involved (14). In general, Th1 cells arise in the presence of IL-12 and promote cell-mediated immunity by producing IFNγ, TNF-β, and IL-2 (14). Whereas, Th2 cells develop in the absence of IL-12 and presence of IL-4, IL-10 and IL-6 and secrete anti-inflammatory/regulatory factors (IL-4, IL-5, IL-13) that act to resolve or down-regulate the inflammatory Th1 response (14). The Th2 component promotes humoral mediated immunity against extracellular pathogens such as parasites (14).

More recently, additional CD4+ T-cell subclasses have been identified and include Th type-17 (Th17) and regulatory T-cells (T-reg). Th17 cells are effector Th cell subsets that secrete pro-inflammatory cytokines, including IL-17 (17). T-reg cells, as their name implies, regulate the response of effector T-cells, including inducing antigen tolerance (14). Several subclasses of T-reg cells have been identified, including naturally occurring ($CD4^+CD25^+Foxp3^+$), or peripherally induced Tr1 (secrete IL-10) or Th3 cells (secrete TGF-β). The exact roles of Th17 and T-reg cells in the immune system have not been fully characterized, but emerging evidence suggests that functional T-reg cells are necessary to prevent autoimmune diseases (14) and other chronic conditions, such as atherosclerosis (21); whereas, Th17 cells are believed to be essential mediators of inflammatory neutrophil responses (23).

1.2.2 T-cells and Inflammation

The inflammatory response to infection is generally a necessary life-preserving response. However, any imbalance or irregularity in the immune system could cause persistent or chronic inflammation that result in excessive tissue damage. T-cells are actively involved in the onset, propagation and resolution of inflammation. Thus, impaired and/or imbalanced T-cell responses have been implicated in the pathogensis of chronic inflammatory diseases (25). Classically, researchers characterized inflammatory autoimmune diseases by a polarized Th1 response (IFN-γ, IL-2 and TNF-β) (25). Although it is likely that a skewed Th1 cytokine response is still involved in chronic inflammation, activated Th17 cells and dysfunctional T-reg cells have also been implicated (17, 28). In addition, the interaction between APCs (via MHC class II molecule) and T-helper cells dictate the type of immune response that is generated (14). Thus, abnormalities in APCs, including macrophages and dendritic cells, likely contribute to impaired T-cell responses in chronic inflammatory states (17). Regardless of the exact cause of autoimmune disease, it is clear that T-cells contribute significantly to the onset, propagation and resolution of inflammation.

1.2.3 *Ex vivo* Measures of T-cell Function

There are several ways to determine the function of T-cells *ex vivo*, including estimating the proliferative response of T-cells and their ability to produce cytokines in response to mitogen or antibody stimulation. The most commonly used T-cell mitogens

are concanavalin A (ConA), phorbal myristate acetate + ionomycin (PMAI), and phytohemaglutinen (PHA). ConA stimulates T-cells via the T-cell receptor (32), PMAI (34, 35) directly activates PKC and PHA binds to glycoproteins including the T-cell receptor (36). Pokeweed mitogen (PWM) is a plant lectin and activates both B- and T-cells (37). Lipopolysaccharide (LPS) is an endotoxin derived from E. coli that stimulates B-cells (39) and macrophages via the toll-like receptor 4 (TLR-4) (40). The response to antigenic peptides can also be measured if the subject or animal has been exposed *in vivo* (42). Proliferation, or the ability of cells to divide, is typically measured by the incorporation of tritiated (^{3}H) thymidine into the DNA of propagating cells (42). The ability and pattern of cytokine secretion by immune cells in response to stimulation is also a useful estimation of immune cell function (42).

1.2.4 Gut-Associated Immunity

Gut-associated lymphoid tissue (GALT) is a major site of antigen sampling and believed to be the most important location for the induction of tolerance to dietary and microbial antigens. It functions primarily to allow non-pathogenic substances, such as commensal bacteria, to survive and likely plays a major role in the tolerance to food antigens, while protecting the host from pathogenic organisms and other potentially toxic substances (43). GALT is a component of mucosal immunity and is composed of aggregated tissue including Peyer's patches and solitary lymphoid follicles, and non-aggregated cells in the lamina propria and intraepithelial regions of the intestine, as well as mesenteric lymph nodes (MLN) (43). Recognition of oral antigens occurs in the MLN when dendritic cells migrate to the nodes and interact with resident T-cells (44). As such, the MLN are believed to be the primary site of oral tolerance (44).

1.3 Obesity and Immune Function

1.3.1 Human Studies

Several research groups have identified impairments in immune function in the overweight population, including both the innate and acquired branches of immunity. Although there is a general consensus that immunity is impaired, specific abnormalities and the underlying mechanisms remain unknown. For a complete summary of all related studies, refer to Table 9.1, pg. 197 in the Appendix.

1.3.1.1 Inflammation and Obese Humans

The most consistent evidence has been derived from studies that assess the level of various blood inflammatory markers. The current literature reveals that overweight and obese individuals have elevated blood levels of several inflammatory markers, including C-reactive protein (CRP) (45-50), TNF-α (47, 48, 52-56), soluble TNF-receptor (sTNFR)-1 and TNFR-2 (52, 55, 57), IL-6 (47, 49, 56, 58-60) and IL-1β (56). A smaller number of studies have reported no differences in blood concentrations of TNF-α (49, 58, 59), IL-1β (59) and IL-6 (50, 61) or even decreased TNF-α (61) concentrations in obese individuals. The majority of studies available strongly suggest that obesity is a chronic inflammatory state and imply that abnormalities in the immune system are present. Impairments in the both immune system and adipose tissue have been implicated as major contributors. The following sections in this review focus on what is known about immune function in the obese population.

1.3.1.2 Epidemiological Studies

Few studies have attempted to characterize immune-related impairments in obese individuals. However, population-based studies have served to identify several potential immune related conditions associated with the accumulation of excessive fat mass. An increased incidence of infection in obese patients has been identified after surgical procedures, including prosthetic (i.e. hip arthroplasty)(5, 62), cardiac (63-67), spinal (68, 69), vaginal (70), and caesarean section (71). Obese patients are also reported to have a higher mortality rate following surgery (65, 72). In addition to hospital-acquired infections, the incidence of periodontal disease (73, 74), respiratory, urogenital, and cutaneous infections as well as bacteraemia (75) have been reported to be higher in obese individuals (as reviewed by (76)). Collectively, population-based studies indicate that individuals with a high BMI are at greater risk of surgical and non-hospital related infections and are more likely to succumb to these infections. This suggests some level of immune suppression accompanies the obese state.

1.3.1.3 Atopic Disease and Obesity

In the past few decades the incidence of atopic diseases, defined by skewed Th2 responses, has escalated dramatically, particularly in Westernized countries (77). Concurrently, obesity rates have also risen over the past few decades leading researchers

to speculate that impaired immune responses in the obese state might contribute to development of asthma and allergic disease (77). A recent meta-analysis of prospective cohort studies reported that as compared to a normal BMI (<25 kg/m^2), individuals who are overweight (>25 kg/m^2, but ≤29.9 kg/m^2) or obese (>30 kg/m^2) were at higher risk of developing asthma (OR=1.51, 95% confidence interval, 1.27-1.80) (78). Furthermore, there was an incremental increase in risk as BMI increased; those who were obese had a nearly two-fold (OR=1.92, 95%CI, 1.43-2.59) increased likelihood of developing asthma compared to lean individuals (78). There is also evidence to suggest that there is a greater prevelance of atopy with a higher BMI (79-81). The increased prevalence of asthma and atopy in the overweight population is suggestive of an overactive Th2 response and thus further suggests impaired T-cell function in obesity.

A recent meta-analysis also reported that adults with a higher BMI were at greater risk for several forms of cancer, including oesophageal, renal, thyroid and colon, as well leukaemia, multiple myeloma, and non-Hodgkins lymphoma (slightly weaker association) (82). In addition, women with a great BMI had an increased risk of endometrial, post-menopausal breast and gallbladder cancer (82). Currently, the underlying aetiology remains unknown; however, impaired T-cell function (83) and a sustained inflammatory environment contribute to tumour development and progression (84). Thus, altered T-cell function and the inflammatory state of obesity may favour the onset of tissue malignancies. To date, this remains a largely unexplored area, but warrants investigation due to the chronic disease burden in overweight individuals.

1.3.1.4 Acquired Immune Response

Overall, population-based studies point to several aspects of immunity that may be impaired in the obese state. Although research is still very limited in this area, both B and T-lymphocyte function have been reported to be altered in this population.

T-cell Function

There are studies that suggest that T-cell function is altered in overweight people. The proliferative response of T-lymphocytes isolated from obese adults was lower when cells were stimulated *ex vivo* with ConA or PHA (52, 85, 86). The reduced proliferative capacity of T-cells was not likely due to a reduction in the number or proportion of T-cells because Tanaka *et al* (1993 and 2001) (52, 85) used isolated T-cells and Nieman *et al* (1999) used whole blood which they reported a higher proportion of T-cells. In addition, there is some evidence to suggest that T-cell function may be impaired in obese children, although the literature is conflicting (56, 87, 88). Overall, the limited data available does support that with increasing body mass there is a decrease in an individual's ability to mount an adequate acquired immune response. However, these studies did not report other lifestyle characteristics, such as diet or physical activity, which have also been shown to impact immunity. To date, no one has attempted to explore the underlying mechanisms involved in the impaired responses of B and T-lymphocytes in obese individuals.

Humoral Immunity

An early study conducted by Weber *et al* (1985) (89) suggested that obese adults had impaired humoral immune responses. In response to Hepatitis B vaccines, individuals with higher BMIs had poor antibody titres (89). Since this report was published, one other group has reported that obese children also had poor antibody titres in response to Hepatitis B vaccine (90). Furthermore, low IgG anti-tetanus concentrations were reported in obese children and adolescents following tetanus vaccination (59). Altered serum concentrations of salivary IgA and C3c (complement factor) (91) and IgE specific to a group of common allergens (81) have also been reported, lending further support that the humoral immune system may be suppressed by the accumulation of excessive fat mass and/or other factors associated with obesity such as micronutrient status or food intake. Moreover, the proliferative response, as measured by ^{3}H-thymidine uptake, of pokeweed mitogen (PWM) stimulated B-cells was lower in obese subjects (85, 86). To date, studies have not examined the underlying mechanisms involved in these responses. However, it is likely that impairments in B-cell function are due to cell dysfunction as opposed to the number of B-cells present in the blood. Studies indicate that the concentration of B-cells

in the blood do not differ between lean and obese subjects (52, 87); in fact, Nieman *et al* (1999) (86) reported that blood concentrations were actually elevated. Overall, it appears that the humoral immune response in obese humans is impaired, but further research is required to understand the molecular mechanisms involved. In addition to the obese phenotype, it is likely that nutrient status and dieting behaviour could contribute to the impaired immunity.

1.3.1.5 Innate Immune Response

Although there is strong evidence that obese individuals have higher circulating markers of inflammation, the function of innate immune cells has been poorly studied. Tanaka *et al* (2001) (52) is the only group to report that monocytes isolated from obese humans produce more TNF-α in response to *in vitro* LPS-stimulation. Ghanim *et al* (2004) (47) also reported that there was increased mRNA expression of TNF-α, IL-6, MMP-9 (matrix metallopeptidase 9), and monocyte inhibitory factor (MIF) in freshly isolated blood mononuclear cells of obese subjects. In addition, Ghanim *et al* reported that the higher mRNA expression of inflammatory mediators was likely due to increased DNA binding activity of NF-κB (nuclear factor-kappa B) and decreased Iκ-Bβ (cytosolic inhibitor of NF-κB) protein levels (47). Collectively, these studies indicate that immune cells isolated from overweight individuals may produce more pro-inflammatory cytokines after stimulation and indicate an altered regulation in the obese state.

A growing body of literature has identified leptin as a key mediator of the immune system. Functional leptin receptors have been identified on innate immune cells, including macrophages (92) and dendritic cells (93). Various animal studies and cell culture experiments suggest that leptin affects the ability of such cells to produce inflammatory cytokines (as reviewed by (94)). Due to leptin's role in stimulating immune cell function and the fact that leptin resistance appears to be an integral feature of human obesity (95), one could hypothesize that inflammatory immune cells would have a reduced capacity to produce cytokines in response to stimulation. However, of the limited evidence available it appears that innate immune cells have a heightened inflammatory reaction. This suggests that the inflammatory response may be at least partially due to an impaired ability to adequately suppress inflammation.

Abnormalities in the production of inflammatory mediators may also be due to alterations in the proportion of innate immune cells present in blood or *ex vivo* assays. A few research groups have examined the distribution of macrophages and monocytes in the blood of obese humans. It has been reported that overweight individuals have a higher proportion of circulating macrophages/monocytes (86, 96). However, one group has reported that there is no difference in the percentage of blood macrophages/monocytes (97). It is possible that higher blood concentrations of macrophages and monocytes contributed to the higher LPS-induced TNF-α production reported in Tanaka *et al*'s study (52) and the higher NF-κB binding activity and inflammatory mRNA expression in Ghanim *et al*'s study (47). Similarly, the higher circulating levels of inflammatory mediators in obese individuals may be due, at least in part, to higher circulating levels of macrophages and monocytes. However, this does not rule out an effect of the obese state directly on phagocytic cell function.

1.3.1.6 Implications of Impaired Immune Function in Obesity

The rapid incidence in obesity is of serious concern due to the major chronic diseases that are associated with a higher BMI and increased visceral fat. There is a growing body of evidence indicating that a heightened inflammatory state and an impaired immune system are major contributing factors to the development of cardiovascular disease (CVD), insulin resistance, type 2 diabetes, and certain forms of cancer. The following sections briefly discuss the implications of impaired immune responses as they relate to these chronic diseases.

Cardiovascular Disease

Overweight and obese individuals are at greater risk of developing CVD than lean individuals and emerging evidence suggests that inflammation is a contributing risk factor. In fact, inflammatory mediators have been shown to predict the onset of CVD in obese individuals (98, 99). Although these markers are useful predictors, they have also been implicated in the pathogenesis of atherosclerosis. The inflammatory markers that precede CVD are implicated in the etiology of endothelial dysfunction and atherosclerotic plaque development (100). It has been postulated from experimental models that T-cells and macrophages contribute to the development and progression of atherosclerosis (101). Thus, impaired T-cell and macrophage functions that have been identified in obese

individuals may contribute to the onset of CVD, although their exact roles in disease pathogenesis are unknown. It is possible that dietary therapies aimed at improving T-cell function and inflammation could reduce the risk of obese individual developing CVD.

Insulin Resistance and Type 2 Diabetes

The prevalence and severity of insulin resistance is positively correlated with BMI. As such, the incidence of type 2 diabetes is much greater in obese individuals. Inflammation, in addition to other factors, has been directly implicated in the pathogenesis of insulin resistance. It has been well-documented in numerous large scale prospective studies that inflammatory cytokines and acute phase proteins are involved in the progression of insulin resistance to overt diabetes in overweight subjects (102). Furthermore, low levels of anti-inflammatory mediators, such as adiponectin, predict the development of type 2 diabetes in select populations (102). Inflammatory proteins have also been implicated directly in the pathogenesis of insulin resistance and progression to type 2 diabetes (103). TNF-α has been demonstrated to disrupt the insulin signaling pathway by phosphorylating the serine residue on the insulin receptor substrate-1 (103). Signaling pathways involved in the inflammatory response can also interfere with insulin secretion from pancreatic β-cells (103). These observations support inflammatory mediators contributing to insulin resistance and the progression to type 2 diabetes. Therapeutic strategies aimed at reducing the inflammatory response of immune cells might therefore prevent disease progression and improve clinical outcomes in insulin resistant individuals.

1.3.1.7 Summary of Obesity and Immune Function

Overall, obesity is a chronic inflammatory state with poorly defined impairments in immune function. In general, the increased prevalence of infection, asthma and atopic diseases suggest that the immune system of overweight individuals is impaired. However, due to the limitations of human studies the underlying mechanisms are unknown. The most consistent evidence points to altered T-cell function; however, there also appears to be impairments in B-cell responses and potentially proinflammatory innate immune cells. Due to the increased disease burden in the overweight population, focused studies are required to determine the exact immune abnormalities to design targeted interventions.

Although studies conducted in obese individuals are suggestive that increasing fat mass negatively influences immune function, there are a few limitations inherent with these studies. In particular, the influence of diet, eating patterns, sedentary lifestyle and micronutrient status on immunity has been completely overlooked in the existing literature. Recent evidence indicates that obese individuals have poorer nutrient status relative to normal weight adults (104, 105). Lower and/or inadequate serum levels of several micronutrients have been identified in obese individuals, including iron (106), vitamins D (107), A, C, B_6 and E and folate (104, 105). Since most of these nutrients are imperative for immune cell function (108), it is important to consider the nutritional status of obese subjects when assessing immune health. In addition, it is well documented that obese individuals have abnormal eating habits and dieting behaviours (109) and these practices have been shown to negatively influence immune function (110, 111). Overall, in addition to the influence of excessive fat mass, it is likely that poor diet quality, disordered eating practices and nutrient status contribute to the worsened immune responses reported in obese individuals.

1.3.2 Obese Rodents

The limited number of studies conducted in rodent models of obesity suggest that immunity is impaired (refer to Table 9.2, pg. 219 in Appendix for a complete summary of all published articles describing immune function in obese rodents). However due to the lack of comprehensive studies aimed at characterizing immunity, there are many difficulties in interpreting the results. To date, the abnormalities associated with increased fat mass remain poorly defined and there is little agreement as to the aetiology of these immune impairments. One source of inconsistency stems from the lack of a well characterized animal model to examine the impact of obesity on immune function. Some data exists for both genetic and diet-induced models of obesity.

Genetic models of obesity can be monogenic or polygenic, but despite the underlying cause, all models become visibly overweight at an early stage in development. In examining immune function and obesity, the most commonly used monogenic rodent models are the *ob/ob* or *db/db* mouse or *fa/fa* rat. These animal models carry an autosomal recessive form of leptin (*ob/ob*) or a defective long form of the leptin receptor (*db/db* or *fa/fa*) (112). The absence of leptin signalling results in hyperphagia leading to

the onset of obesity at approximately 5-6 weeks of age. Although not used as commonly to study immune function, there are also several polygenic models of obesity, including the spontaneous hypertensive rat (SHR), Otsuka Long-Evans Tokushima fatty (OLETF) rat and Goto-Kakizaki rat (112).

In addition to the *ob/ob* or *fa/fa* rodents, researchers have also studied the effects of obesity on immunity by feeding high fat diets (35-60% of caloric intake) for several weeks to either C57BL/6J mice or Wistar rats. The fat provided in these diets is usually high in saturated fat and control animals are most often fed low fat/low saturated fat chow. It is well documented that the type and amount of fat in the diet influences in the immune system (42).

Thus, it is evident that despite a similar end point, several distinct mechanisms are involved in inducing excessive weight gain and that the discrepancies in these methods may partially account for the variability in the published literature. In the following sections the effects of obesity on immune function in rodent models of obesity will be summarized. Various components of immunity have been examined in rodent models of obesity (see Figure 1.1 for complete summary); however, the following review focuses specifically on aspects of T-cell function and inflammation.

1.3.2.1 T-cell Function

The proliferative response of isolated immune cells has been investigated by several groups using various rodent models of obesity. Collectively, these studies indicate that when the adaptive branch of the immune system is stimulated by PHA, a T-cell mitogen, high fat fed (HFF) obese rodents have reduced proliferative responses (6, 41, 113). Only one study failed to show a difference in PHA-stimulated proliferative response of isolated splenocytes (114). However, this group later found that HFF mice had lower proliferative reactions in a similar study design with a slightly longer feeding period (6). In the genetically obese *fa/fa* Zucker rat, results consistently demonstrate that immune cells isolated from this animal have a reduced capacity to proliferate when cells are activated with ConA *ex vivo* (10, 115, 116). This lower proliferative response was likely partly accounted for by the T-lymphopenia observed in these rodents (115). In contrast, diet-induced models of obesity do not appear to have impaired proliferative responses to ConA (41, 113). More recently, Verwaerde *et al* (2006) (117) examined the

impact of diet-induced obesity on antigen-specific T-cells. T-cells of transgenic mice expressing a T-cell receptor specific to ovalbumin (OVA) had lower *ex vivo* proliferative responses to ConA and OVA when mice were immunized with OVA; but no difference was found in the proliferative response of OVA-stimulated antigen naïve T-cells (117). Overall, studies conducted in both genetic and HFF models of obesity are supportive of an impaired ability of T-cells to proliferate when stimulated by T-cell mitogens or antigen. However, these impairments are specific to the type of mitogen and rodent model used and may be modulated by the high fat diets fed. Further consideration and study is required to determine which model most adequately represents human obesity.

Abnormalities in the acquired immune system of obese rodents are also observed using *ex vivo* stimulated cytokine production. Splenocytes of HFF obese rodents had lower *ex vivo* IL-2 production compared to lean rodents after stimulating with T-cell mitogens or antigen (OVA) (41, 114, 118); although there was no difference with antigen stimulation in orally sensitized HFF mice (118). This suggests that obesity and/or high fat feeding reduces the capacity of T lymphocytes to produce a proliferative cytokine and this is consistent with the lower proliferation reported by others. However, an additional experiment conducted in HFF mice contradicts this conclusion (6). Splenocytes isolated from mice with induced airway hypersensitivity to OVA produced higher levels of IL-2 when cells were stimulated with OVA *in vitro*. Furthermore, these sensitized mice (lard fed group only) also had higher proliferative responses to OVA stimuli (6). This study suggests that when the immune systems of obese mice are sensitized *in vivo*, there may be an exaggerated hypersensitivity response. In summary, the effect of excessive weight and high saturated fat diets on IL-2 production supports the immune cell proliferation data observed in the literature when obese rodents are not exposed to a stimulus *in vivo*. However, orally sensitized mice (fed high saturated fat diets) appear to have a higher hypersensitivity response and further work is required to understand if this is related to obesity or diet.

B-cell/Humoral Immune

- ↓ antibody-forming splenocytes (ob/ob)(1)
- ↓ antigen specific and non-specific antibodies BSA sensitized (ob/ob) (4)
- ↓LPS-stimulated proliferative response (DIO) (6)

NK Cells

- ↓ cell cytotoxicity (*fa/fa* Zucker and DIO) (10, 11)
- % of NK cells ($CD5^-CD8^+$) not different from lean (fa/fa Zucker rats) (10)
- ↓ % of NK cells ($DX5^+CD3^-$) (DIO mice)(11)
- ↓ IL-18 mRNA (required for NK cell activity) expression virally infected lung (DIO mice) (11)

NK T-cells

- ↓ % NK T-cells (liver) (DIO) (15)
- ↑ % of $TNF\text{-}\alpha^+$ or $IFN\text{-}\gamma^+$ cells (DIO) (15)

Neutrophils

- ↓ phagocytosis (ob/ob mice) (16)
- ↓ CD11b expression (ob/ob mice) (16)

LPS-Stimulated Cytokine Response

- TNF-α not different in blood monocytes or alveolar macrophages (DIO mice) (18)
- ↓ TNF-α peritoneal macrophages (DIO mice) (19)
- ↓ TNF-α Kupfer cells (fa/fa Zucker rats) (20)
- ↓ mRNA expression of TNF-α and IL-6 unstimulated splenocytes (DIO rats) (22)
- ↑ IL-12 & IL-15 by Kupfer cells (ob/ob mice) (24)
- No difference cytokine mRNA or activation marker expression of DCs (ob/ob) (26)

Bacterial/Yeast Infections

- ↑ bacterial load (27), bacteremia and mortality (29-31)
- ↑ infectivity of *candida albicans* (yeast) (*fa/fa* Zucker rats) (33)
- ↓ phagocytically active peritoneal and alveolar macrophages (*ob/ob*)(31, 38)
- ↓ bactericidal activity and production of oxygen radicals (ob/ob and DIO rats) (31, 41)
- ↓ bacterial clearance (liver) and killing *in vivo* (ob/ob) (38)
- Normal phagocytosis (DIO rats) (41)

Dendritic Cells

- ↑ % in spleen (ob/ob)(26)
- ↓ IL-10 expression in mature DCs (ob/ob) (26)
- ↓ ability to stimulate T-cells (ob/ob) (26)
- ↓ secretion of IL-4 and IL-10 in presence of ob/ob DCs in mixed lymphocyte reaction (26)
- No difference in IL-12 or IFN-γ secretion in presence of ob/ob DCs in mixed lymphocyte reaction (26)
- ↑ TGF-β in mixed lymphocyte reaction (ob/ob) (26)
- No difference endocytosis capacity (26)

***In vivo* inflammatory cytokine response to infection:**

- ↓ lung mRNA levels of TNF-α, IL-1β and IL-6 3 d post influenza virus A infection (DIO mice) (11)
- Poor resolution of inflammation (sustained levels of TNF-α and IL-6 post-infection) (DIO) (11)
- ↓ TNF-α, IL-6, IL-1β and MIP-2 lung concentrations post-bacterial infection (ob/ob) (19, 51)
- No difference in TNF-α, IL-6 or MIP-2 lung concentrations post-bacterial infection (ob/ob) (27, 29)
- ↑ MIP-2, TNF-α and PGE_2, but no difference in IL-6 lung concentrations post-bacterial infection (ob/ob) (31)

Figure 1.1. Summary of the effects of obesity on immune function excluding T-cell function.

1.3.2.2 Th1 Cytokine Response

The pattern of cytokines secreted in response to stimulation has been used to determine whether T-cells respond in a characteristic Th1 or Th2 response. The Th1 cytokine response is typically characterized by higher IFN-γ secretion, particularly in relation to IL-4, a Th2 cytokine. After careful review of the literature, there is an apparent difference in IFN-γ production in diet-induced versus genetic models obese rodents. Higher IFN-γ production has been reported in PHA-stimulated splenocytes as well as ConA or OVA-stimulated naïve splenic T-cells (OVA-TCR transgenic mice) in obese mice fed high saturated fat diets (114, 117). Furthermore, obese offspring of diabetic dams had higher spleen IFN-γ mRNA and serum concentrations (119). Collectively, these studies imply that there is a heightened Th1 response in naïve cells isolated from HFF obese mice. However, Verwaerde *et al* (2006) (117) and later Mito *et al* (2002) (6) reported that there was no difference in mitogen- or antigen-stimulated splenocyte IFN-γ production in HFF mice sensitized to OVA *in vivo*. This suggests that naïve T-cells (i.e. unexposed to antigenic peptide *in vivo*) have a skewed Th1 response, but this may disappear when diet-induced mice are exposed to antigen *in vivo*. However in a subsequent study, with a longer feeding period these authors (120) did not reported a difference in IFN-γ production after PHA or anti-CD3 stimulated splenocytes with HFF mice. Similarly, *ex vivo* ConA-stimulated IFN-γ was not reported to differ in HFF-obese rats compared to lean chow-fed rats (41). Collectively, studies conducted in diet-induced rodents regarding the Th1 cytokine, IFN-γ, are conflicting; discrepancies in results may be due to the maturation state of immune cells examined, the type of stimulus used or the amount and type of fat in the diets used to induce obesity.

In contrast to studies conducted in diet-induced models of obesity, examination of Th1 responses in leptin deficient mice fed a chow diet have yielded more consistent results. With respect to genetic models of obesity, it has been reported that *ob/ob* mice produced less IFN-γ in models of contact hypersensitivity (121) and arthritis (4) and in response to tuberculosis infection (27). In addition, *ex vivo* IFN-γ production in response to tuberculin protein stimulation was lower in *ob/ob* mice (27). Thus, the lack of a leptin in the *ob/ob* mouse may contribute to the inability to mount an adequate Th1 cytokine response. Indeed, researchers have shown that restoring leptin levels in *ob/ob* mice

normalized the ability of immune cells to produce IFN-γ (27, 122). From the literature there appears to be differences in the immune defects reported using genetic vs. diet-induced models of obesity, suggesting that the complete absence of leptin or feeding a high saturated fat diet to induce obesity alters the ability of T-cells to produce an important Th1 cytokine.

1.3.2.3 Th2 Cytokine Response

Th2 cells are characterized by secretion of IL-4, IL-5 and IL-13. This branch of the acquired immune system is involved in stimulating the humoral immune response, including activation of B-lymphocytes, antibody production and IgG to IgE class switching (14). An overzealous or dysregulated Th2 response is believed to be involved in atopic diseases and recent evidence suggests that obese individuals are at greater risk for atopic disorders (77). However, there are few studies assessing the ability of T-cells to produce Th2 cytokines, particularly IL-4, in response to mitogen stimulation in rodent models of obesity. Mito *et al* (2000) reported that PHA-stimulated splenocytes from HFF mice produced more IL-4 *in vitro* (114), suggestive of a skewed Th2 response. However, obese rodents that were challenged *in vivo* with a bacterial pathogen or *ex vivo* with an antigenic peptide, did not differ in the amount of IL-4 produced (27). Furthermore, Mito *et al* (2006) (118) later reported that there was no difference in IL-4 production of splenocytes isolated from OVA sensitized HFF mice. In summary, using the limited evidence available, one might conclude that diet-induced obese mice have a tendency to produce more IL-4 in response to mitogen stimulation. However, when obese mice are challenged with a specific infection or antigenic peptide, splenocyte IL-4 production does not appear to be abnormal. Further investigation using more comprehensive approaches is required to understand the impairments involved in eliciting atopic or Th2-skewed diseases in obese states.

IL-10, formerly classified as a Th2 cytokine, is a key regulatory cytokine that is secreted by CTLs, Th1, Th2, regulatory Th17 and regulatory T-cells (123). Acting at the interface between antigen presenting cells and T-cells, IL-10 controls the host response to foreign antigen and the Th1 inflammatory reaction (123). Despite the importance of this cytokine in regulating T-cell and inflammatory responses, there is little information on its production in the obese state. Lamas *et al* (41) (2002) reported that ConA stimulated

production of IL-10 was similar in diet-induced obese rats compared to lean control rats. Similarly, Wieland *et al* (2006) (51) later reported that *ob/ob* mice had similar lung concentrations of IL-10 to lean mice after a challenge with *S. pneumoniae* and *K. pneumoniae*. However, Smith *et al* (2007) (11) noted that lung mRNA expression of IL-10 was lower shortly after influenza A infection in high fat/high sucrose-fed mice, but levels did not differ 6 days post-infection. Overall, studies conducted thus far suggest that the obese state, regardless of the method of induction, has a limited impact on the production of this key immunoregulatory cytokine.

1.3.2.4 Dendritic Cell Function

Dendritic cells (DCs) are cells of the innate immune system that present antigen to and activate naïve T-cells. DCs play a major role in inducing the development of Th1, Th2 or T-regulatory cell immune responses through secretion of cytokines and ligand-mediated interactions. Despite such an important role in regulatory and effector T-cell development, the impact of obesity on DC function has not been clearly established. Macia *et al* (26) observed higher proportions of DCs in the spleen of *ob/ob* mice. Further functional investigations revealed that LPS-stimulated bone-marrow derived DCs (BM-dDCs) of *ob/ob* mice had a similar expression of activation markers (i.e. CD40, CD80 or CD86) and similar endocytic capacities compared to lean mice (26). However, mature BM-dDCs had a lower ability to stimulate T-cells to proliferate and to produce IL-10 and IL-4 in an allogenic mixed lymphocyte reaction. The authors further postulated that the lower proliferative response of T-lymphocytes may be due to the substantially higher production of TGF-β. Other researchers have shown that production of TGF-β by DCs induces the development of regulatory T-cells, which respond poorly to stimulation (124). In summary, dendritic cell function is reported to be impaired in one genetic model of obesity. Clearly, further work is required to determine if similar impairments are present in other models of obesity and humans.

1.3.2.5 *In vivo* Models of Inflammation

The effects of obesity on inflammatory arthritis have been examined in leptin-deficient mice; however, the results are not consistent, likely due to the different methods employed to induce arthritis. Busso *et al* (2002) (4) reported that *ob/ob* mice were protected from bovine serum albumin (BSA)-induced joint arthritis and had lower

synovial tissue levels of IL-1β and TNF-α. However, Bernotiene *et al* (2004) (125) later reported that there was increased cartilage damage and inflammatory infiltration in the knee joint of these animals following zymosan A injection. Although, there were no differences in inflammatory cytokine mRNA in the inflamed joint, obese mice had higher circulating levels of IL-6 and serum amyloid A (125). Bernotiene *et al* also reported that there was a greater acute phase response in *ob/ob* mice and resolution was delayed (125). The likely cause of these differing results between these two studies is the methods used to induce arthritis. The antigen-induced (BSA) method requires involvement of the adaptive immune system to induce tissue damage; whereas the zymosan A method directly induces arthritis via binding to toll-like receptor 2 (TLR-2) independent of leptin and the acquired immune system (125). The authors postulate that the lack of inflammatory response and damage in the first study was due to impairments in T- and B-cell immune responses in the *ob/ob* mouse (125). Thus, it appears that *ob/ob* mice may be protected from allergen induced arthritis due to B- and T-cell impairments, but may be more susceptible to inflammatory joint damage when innate immune cells are activated via TLR-2.

1.3.2.6 Allergy

Allergy or a type I hypersensitivity reaction is an immune response to an innocuous substance, defined as an allergen (126). This process involves humoral mediated immunity, including B-cell and Th2 driven responses (126). There has been a marked increase in the incidence of allergic diseases over the past few decades concomitant with a rise in the occurrence of obesity (77), suggesting an association. Consistent with this hypothesis, there appears to be a greater incidence of allergy and asthma in the overweight population (77). In an animal model of antigen-induced asthma (OVA-sensitized), Mito *et al* (2002) (6) observed that obese, HFF mice produced more IL-2 and proliferated more in response to OVA *ex vivo*. Furthermore, there were a greater number of mast cells in the tracheal mucosa (6). However, the lower serum concentration of OVA-specific IgE suggests that the higher response in obesity to antigens may be due to the greater sensitivity of antigen-specific T-cells rather than via an IgE mediated pathway (6).

1.3.2.7 Delayed-type or Type IV Hypersensitivity Reactions

A delayed-type hypersensitivity (DTH) reaction is a local inflammatory response initially induced by Th-cells to specific antigens. Although other non-specific inflammatory cells mediate the DTH response, macrophages are the primary effector cells (13). Two studies have attempted to examine the effect of obesity on type IV hypersensitivity reactions, one in a diet-induced model of obesity and the other in leptin-deficient mice. Following topical sensitization to a skin-reactive hapten (2,4,6-trinitrochlorobenzene), obese mice had less ear swelling and lower IFN-γ levels in draining lymph nodes in response to the hapten (121). Furthermore, transfer of immune cells from topically sensitized obese mice to lean mice did not induce ear swelling that cells of sensitized lean mice did (121). The lower level of IFN-γ and IL-4 produced by cells in the lymph nodes (anti-CD3-stimulated) was also indicative of an impaired contact hypersensitivity reaction in diet-induced obese mice (121). Katagiri *et al* (2008) (121) also examined protein-adjuvant (OVA with complete freund adjuvant) hypersensitivity reactions and noted that there was no difference in the production of OVA-stimulated IFN-γ or antigen-specific IgG1 and IgG2a/2b from splenocytes of OVA-sensitized obese mice (121). In agreement, Wieland *et al* (2005) (27) noted that the DTH response to tuberculin protein in *ob/ob* mice was lower, as measured by foot pad thickness and inflammatory infiltrate. Together, these studies provide evidence that obese rodents, both diet-induced and genetic, have impaired type IV hypersensitivity reactions. However, Katagiri *et al*'s study suggests that high-saturated fat fed obese mice have normal protein-adjuvant hypersensitivity reactions.

1.3.2.8 Oral Tolerance

Oral tolerance is the process by which immune cells, particularly T-cells, respond to an antigen in a manner that prevents a proliferative response. Encounter of a small dose of an antigenic peptide in the GALT typically induces T-reg cells to respond in a manner that suppresses reactivity upon future encounter of this antigen (127). Oral tolerance in obesity has been investigated in a diet-induced mouse model. Splenocytes of antigen-naïve obese mice produced less IL-2 than lean control mice, but IL-2 production did not differ between lean and obese orally sensitized mice (118). Furthermore, serum concentrations of IgG1 and OVA-specific IgA and IgM were lower in orally sensitized

obese HFF mice relative to lean, low fat-fed controls (118). The authors imply that the lower concentration of IgA may render obese rodents less tolerant to oral antigen challenge (118). Overall, the study implies an impaired ability to develop tolerance to oral antigens in obese states, which is supportive of the increased prevalence of allergic diseases in obese adults (77). A major limitation to these studies is the failure to examine the gut-associated immune system or to consider the impact of high saturated fat diets and warrants further examination.

1.3.2.9 Gut-Associated Immunity and Visceral Adipose Tissue

There is emerging evidence that obese individuals have altered gut microflora (128) and thus potentially altered antigen exposure, which may influence gut-associated immunity (129). MLN are situated amongst the inflammatory environment of visceral adipose tissue, which likely affects resident lymph node cells (130). Visceral adipose tissue can produce and secrete a variety of factors, including free fatty acids and inflammatory mediators that have been implicated in the pathogenesis of insulin resistance and other chronic diseases (131). Despite the potential influence that adipose tissue mediators could have on lymph node cell function, only one study has addressed this. Kim *et al* (2008) (132) reported that HFF mice had smaller MLN with fewer immune cells compared to chow-fed mice. This lymphopenia affected the T-cells (CD4+ and CD8+), regulatory T-cells ($CD4^+CD25^+Foxp3^+$), $CD4^+CD25^+$, neutrophils, dendritic cells and B-cells (132). However, despite the lower concentration of lymphoyctes, there were greater numbers of activated, mature T-helper and T-cytotoxic cells in MLN from obese HFF mice (132). Based on studies conducted in lean mice, the authors hypothesized that factors emanating from the mesenteric fat, such as free fatty acids and H_2O_2, induced apoptosis of lymphocytes residing in MLN and thus decreased the cellularity (132). Overall, this study is suggestive of impaired gut-associated immunity in HFF mice.

1.3.2.10 Limitations of Current Studies

While animal models allow us to examine the effects of dietary intervention on tissues and organs to a greater extent than human studies, limitations exist in the use of these rodent models. The leptin-deficient *ob/ob* mouse and leptin-receptor deficient *fa/fa* Zucker rat have been used as a model of obesity by a number of researchers. The

identified shortcomings of using these models are that leptin is a key mediator of both T-cell and inflammatory immune reactions (94) and these genetic defects are rare in overweight/obese humans (133-136). However, leptin resistance is an integral feature of obesity (95) and therefore, likely contributes to the immune dysfunction of human obesity.

In obesity research comparisons are also commonly made between rodents fed high-fat diets composed mainly of lard to rodents fed low fat chow diets. It is well documented that both the type and amount of fat in the diet can influence almost every aspect of immunity (42). The main criticism of this rodent model of obesity is that the composition of the experimental diets used to induce obesity varies considerably from the composition of the typical North American diet. Total fat accounts for approximately 33% of energy consumed in US diets (137) compared to 40-70% of energy provided in high fat rodent diets. Chow diets contain a very low level of fat ranging form 5-7% w/w fat or 13-15% of calories (138). It is estimated that dietary intake of saturated fat in the US is 33% of total fat (139), while lard contains approximately 46% saturated fat (140). High fat diets suppress immunity, including hypersensitivity responses (141, 142), surface expression of CD3 and CD25, (143), NKT cell function and Th1 responses (144) and antibody production (142) compared to low fat diets with similar (141-143) or unknown fat composition (144). To a lesser extent, highly saturated fat diets have also been shown to influence delayed-type hypersensitivity reactions (145) in healthy subjects and can induce macrophage inflammation and disrupt antigen presentation *in vitro* (146, 147). In rodent studies, the high saturated fat diets that have been used, contain low levels of the essential (n-3) PUFA and lack a source of longer chain polyunsaturated fatty acids, which may also negatively impact the immunological parameters measured. It is well established that insufficient levels of essential dietary fatty acids can be immunosuppressive (148).

In addition to the potential confounding effects of dietary composition and genetic defects, many of the studies discussed in the previous sections used very young rodents. Immune responses vary considerably from post-weaning periods to adulthood (149). Thus, examination in early stages of development could impact extrapolation to adult humans. Moreover, young rodents do not develop CVD, a major complication of

obesity and insulin resistance in which inflammation and possibly T cell dysfunction contributes to disease pathogenesis. The age of animal studied should be taken into consideration when designing future studies.

Collectively, the studies reviewed above indicate that obesity is characterized by impaired immune responses. However, the underlying mechanisms are poorly understood and several inconsistencies are evident in the literature. These may be due to the animal model (diet-induced vs. genetic) and the age of the rodents used. There are shortcomings associated with both types of obesity models. However, it would be beneficial to identify a model that best represents the immune abnormalities associated with obese humans. The majority of studies conducted in genetic models have used chow diet or a diet that does not contain a fat level or composition that is consistent with that consumed by the North American population. This should be considered in future study designs.

1.4 Diet and Obesity

Obesity is a complicated, multi-factorial disease in which both environmental and genetic factors contribute to development (150). Regardless of the initiating or contributing factors, obesity is a state defined as an imbalance between energy intake and energy expenditure (151). Several nutrients and foods are currently being examined for their weight gain or loss-promoting properties. However, it is unlikely that a single nutrient is solely responsible for the obesity epidemic. Dietary fat intake has been identified as a potential contributor to the obesity epidemic (152). Traditionally, caloric restriction and lower fat diets (25-30% of calories) were integral treatments for reducing excessive fat mass (153). Caloric deprivation influences many parameters of immune function (110). More recently, low carbohydrate, high protein diets that tend to be higher in saturated fats have gained popularity and have been demonstrated to assist in weight loss and improve some of the co-morbidities associated with obesity (154). The effect of these diets on immune dysfunction in obesity is not known. Although initial weight reduction has been successful, for both low fat and high protein diets, long-term weight management has proven difficult (153). The current dietary interventions for obesity have not been specifically aimed at improving immune abnormalities, nor has this been addressed beyond plasma inflammatory markers. More information on the effects of

current dietary interventions in obesity aimed at weight loss, such as CLA, is needed. Information on the potential benefits of dietary fat manipulations directly aimed at modifying immune dysfunction in the obese state, such a long chain n-3 PUFA, are needed.

1.5 Dietary Fat

1.5.1 Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is a collective term that refers to a class of positional and geometric conjugated dienoic isomers of LA. The most abundant, naturally occurring isomer, cis-9, trans-11 (c9t11), is as an intermediate species in the biohydrogenation of LA to vaccenic acid in the rumen (155) and is also converted from vaccenic acid by Delta-9 desaturase in the mammary gland (156). Thus, dairy and beef products are the main source of this isomer in the diet (155). CLA can also be produced synthetically from various oils that are rich in LA (e.g. safflower oil, canola oil, soy oil) by alkaline isomerization reactions (155). Synthetically prepared CLA mixtures are composed of many isomers, but c9t11 and trans-10, cis-12 (t10c12) usually comprise the majority of isomers (155). Dietary intake of c9t11 CLA was estimated to be 95 mg/d (157) in Canada; estimated total intake of CLA in other Westernized countries ranges from 74-323 mg/d (158-162). Since its identification as an anti-carcinogenic nutrient, many potential health benefits have been attributed to CLA, including immune modulation. To date, no one has investigated its effects on immune cell function in obesity.

1.5.2 Polyunsaturated Fatty Acids

Dietary essential fatty acids consist of the polyunsaturated fatty acids (PUFA), linoleic acid (LA, 18:2(n-6)) and α-linolenic acid (ALA, 18:3(n-3)). ALA can be converted to eicosapentaenoic acid (EPA, 20:5(n-3)) and then via two different pathways to docosahexaenoic acid (DHA, 22:6(n-3)) by a series of elongation (addition of 2 carbon atoms per step) and desaturation (addition of a *cis* double bond) events in mammalian tissues. However, this process is widely considered to be very inefficient, particularly when the dietary (n-6):(n-3) PUFA is high (163). The dietary reference intakes for essential fatty acids in Canada and the United States recommend that (n-6) fatty acids (LA) should represent 5-10% of and 0.6-1.2% of energy should be (n-3) ALA, of which

up to 10% can be EPA and DHA (164). The ideal ratio of (n-6):(n-3) PUFA in the diet has not been established by the National Academy of Science; but the International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommends that healthy individuals should consume 2% of energy as LA and 0.7% of energy as ALA with an additional 500 mg/d EPA and/or DHA, making the (n-6):(n-3) closer to 2:1 (165). However, evidence suggests that a lower (n-6):(n-3) PUFA ratio may be beneficial in chronic disease states and the American Heart Association currently recommends that individuals with CVD consume 1 g/d of EPA and DHA (166). The most recent report from the US National Health and Nutrition Examination Survey (1999-2000) estimated that LA intake was 14g/d and ALA was 1.4g/d (139); while levels of dietary EPA and DHA are much lower at 30 and 70 mg/d, respectively (139). Our richest dietary sources of ALA include canola oil, flaxseeds and/or oil and walnuts (167); whereas, EPA and DHA are found most abundantly in fatty cold water fish and have been added in smaller amounts to other food sources (167).

1.6 Dietary Fat and Immune Function

It is well established that the type and total amount of dietary fatty acid can influence immunity throughout the lifespan and under various disease states (42). Higher fat diets can impair cell-mediated immunity and modifying total dietary fat intake from high to moderate levels has been shown to improve T-cell proliferative responses and NK-cell activity (42). In addition to total dietary intake, individual fatty acids can to influence the inflammatory response and T-cell function. In particular, EPA and DHA have garnered substantial attention for their ability to modulate various parameters of immunity in both healthy and diseased states. More recently, CLA has been shown to modulate inflammation and acquired immune responses.

1.6.1 CLA and Immune Function

Several studies have reported the effects of CLA, both c9t11 and t10c12 isomers, on several aspects of immunity in cell culture systems, animal models and human trials. Experimental evidence indicates that the various CLA isomers have beneficial effects (albeit not necessarily the same effects) on atherosclerosis, body composition and systemic inflammation (168, 169), suggesting that CLA supplementation may be beneficial in obesity. However, with the exception of studies that have investigated the

effect of CLA isomers on systemic markers of inflammation, there are very few published reports regarding its effects on immune cell function in obesity. Refer to Table 1.1 for a general summary of the reported immune modifying properties of CLA.

1.6.1.1 Intervention Studies

To date, cell culture work and animal studies have dominated the literature, providing much stronger and more convincing evidence than human intervention trials regarding immune function. Supplementation studies conducted in healthy individuals have not generated data that demonstrate that feeding CLA can significantly influence immune function. In healthy, lean adults supplementation of 1.6-3.9g/d of a CLA mixture has been reported to have no effect on numerous immune parameters (170-172). However, others have reported that CLA mixtures increased antibody-specific responses to hepatitis B vaccination (173) and had higher plasma IgA and IgM and lower plasma IgE (174). In addition, T-cell function has been reported to be altered in healthy, lean adults. Those fed a mixture of CLA isomers have been reported to have greater PHA-stimulated IL-2 and TNF-α production (175); while others reported lower delayed-type hypersensitivity reactions (174) and lower ConA-stimulated IL-2 production (176, 177). Furthermore, those supplemented with CLA produced less TNF-α and IL-1β and more IL-10 in response to LPS (174). Overall, CLA supplementation studies conducted in healthy, normal weight adults have produced varying results, but do suggest that CLA can modify T-cell function and inflammatory cytokine production.

The majority of studies examining CLA's immune modulating properties have been conducted in healthy subjects. Recently, Turpeinen *et al* (2008) (178) examined the effects of this fat on immune function in subjects with allergy. Adults with known birch pollen allergy were supplemented with 2 g/d CLA mixture for 8 weeks prior to allergen season and 4 weeks following (178). Immune cells from supplemented subjects had lower concentrations of cytokines (TNF-α, IFN-γ and IL-5) in response to allergen *in vitro* compared to placebo (178). Thus, this study suggests CLA may have beneficial effects in states with skewed Th2 responses.

The majority of the studies examining immune modifying effects of CLA have focused on systemic markers of inflammation in adults. However, interventions conducted in obese men reported that 3.4-4.2 g/d of a CLA mixture or 3.4 g/d t10c12

CLA for 12 weeks in overweight adults did not alter blood concentrations of TNF-α, IL-6, soluble TNF-receptor 1 and 2 or soluble vascular adhesion molecule 1 (179, 180). Furthermore, 3 g/d of c9t11 or t10c12 CLA for 13 weeks did not modify LPS-stimulated cytokine production of blood mononuclear cells or whole blood or serum CRP concentrations (181). However, supplementation with 3g/d t10c12 CLA or 4.2 g/d CLA mixture significantly increased CRP serum concentrations (179, 180) and supplementation of 3g/d t10c12 or c9t11 CLA resulted in higher urinary markers of lipid peroxidation (8-iso-PGF_2) and inflammation (15-keto-dihydro-$PGF_{2\alpha}$) (179, 182), suggesting a negative effect of consuming this fatty acids in those with metabolic syndrome.

Collectively, human intervention studies offer some evidence that CLA can beneficially modulate inflammation and immune function in healthy individuals and those with allergies. However, the limited studies in overweight/obese insulin resistant adults suggest that CLA has negligible effects on immune cell function and inflammation and may even increase an inflammatory risk factor for CVD. However, three of the four studies did not collect diet information nor was dietary intake controlled for during the study periods (179, 181, 182). Furthermore, baseline CRP levels appeared to be higher in the CLA group (3.27 mg/ml) compared the control group (1.76 mg/ml) in Smedman *et al's* (2005) report, which may have influenced the impact of CLA on this measure (180). Thus, more comprehensive studies are required in which dietary intake and baseline inflammatory status are considered to fully understand the influence of the individual CLA isomers on inflammation and immune cell function in obesity and insulin resistance.

1.6.1.2 Animal Studies

To my knowledge, there has been no published report of feeding CLA isomers on immune cell function in rodent models of obesity. Studies conducted in healthy animals or models of inflammation indicate that CLA can modify various aspects of immunity, including T-cell function and inflammation. Our group has published a comprehensive review on this topic (183). The following sections describe aspects of immunity that are modulated by CLA supplementation and provide evidence that these isomers may be beneficial in obesity. Refer to Table 1.1 for a general summary.

1.6.1.3 T-cell Function

The impact of CLA supplementation on the ability of T-cells to proliferate and produce IL-2 in response to mitogen-stimulation has been reported in healthy rodents. Feeding a mixture of CLA isomers increased immune cell proliferative responses to polyclonal T-cell mitogens (184, 185). In support, healthy rodents fed a mixture of CLA isomers had higher IL-2 production in ConA-stimulated splenocytes (184-186); while one group reported that IL-2 production was not altered with CLA supplementation (187). This latter study has been the only to report that the proportion of $CD4^+$ T cells was lower with t10c12 supplementation and $CD8^+$ T cells were higher with the c9t11 or a mixed isomer diet (187). Furthermore, mice fed either t10c12, c9t11 or mixed CLA isomer diet had a lower ratio of $CD4^+$:$CD8^+$ T cells (187). Other key T-cell cytokines have been shown to be affected by CLA supplementation. It has been reported that rodents fed CLA mixtures had higher LPS-stimulated (*ex vivo*) IFN-γ, IL-4, and IL-10 and lower IL-12 (188), while one group reported lower ConA-stimulated IL-4 production (186). Overall, these studies suggest that supplementation with CLA mixtures can increase T-cell responses. The impact on *ex vivo* T-cell cytokine production is less convincing, but suggests that a mixture of CLA isomers can modify responses in healthy, lean rodents.

1.6.1.4 Inflammatory Cytokines

The anti-inflammatory properties of CLA isomers have been examined in feeding studies with healthy rodents with variable outcomes. While some have reported lower mitogen-stimulated inflammatory cytokine production with feeding a CLA mixture or t10c12 alone (187, 188), another group reported no difference (185). Furthermore, Yamasaki *et al* (2003) (187) reported that ConA-stimulated TNF-α production was higher in mice fed c9t11 CLA isomer. Based on these limited studies, it is difficult to draw any definitive conclusions regarding the ability of CLA to alter *ex vivo* inflammatory cytokine production of cells isolated from healthy rodents. However, supplementation studies conducted in models of acute inflammation indicate that CLA supplementation may be beneficial. In a model of bacteria-induced colitis, a mixture of CLA isomers limited mucosal damage and normalized serum IFN-γ and IL-10 concentrations and lymphocyte subset distributions (i.e., $CD4^+$ and $CD8^+$) similar to non-infected pigs (189). Furthermore, a mixture of CLA isomers lowered inflammatory cytokine plasma

concentrations and tissue mRNA levels in a model of acute inflammation induced by LPS injections (190). It was postulated in both studies that the improved inflammatory response induced by CLA was due to greater expression of peroxisome proliferator activated receptor-gamma (PPAR-γ) (189, 190). Collectively, these studies indicate that feeding mixtures of CLA isomers is beneficial during acute episodes of inflammation. Thus, it is logical to hypothesize that CLA may improve the inflammatory immune response observed in obesity.

Table 1.1. Summary of the effects of CLA on various immune parameters

IMMUNE FUNCTIONS	REFERENCE
Reduce immune-mediated catabolism	(186, 190-192)
Improve response to bacterial Ag	(186, 189, 193-195)
Improve the response to viral challenges	(173, 196)
Reduce allergen-induced immune responses	(197, 198)
Reduce the production of pro-inflammatory mediators	(199-206)
Increase the production of anti-inflammatory cytokines	(188)
Potential benefits to acquired immune system	(207, 208)

Adapted from Ruth *et al* (183).

1.6.1.5 Limitations

Overall, animal supplementation studies provide evidence that feeding CLA can modify several aspects of immunity in both healthy and inflammatory states. The mechanism for the effect of CLA on immune function has not been established. A limited number of studies indicate that the two major isomers of study, c9t11 and t10c12, may have different effects on various immune parameters. The available animal studies suggest that feeding CLA could beneficially modify immunity in inflammatory states such as obesity and insulin resistance. Although the sole human study failed to demonstrate an effect of CLA on LPS-stimulated cytokine production, the authors did not

to control or report dietary intake, which likely influenced the immune parameters measured (181). Furthermore, it is unknown how CLA-supplementation affects T-cell function in overweight/obese, insulin resistant subjects.

1.6.2 Long Chain (n-3) PUFA and Immune Function

It is well established that feeding EPA and DHA can modify immune function in animals and humans (209, 210) and there is evidence for many different mechanisms (211). In addition to research in healthy individuals, evidence suggests that supplementation with EPA and DHA can reduce the inflammatory response and improve clinical symptoms in patients with rheumatoid arthritis and other inflammatory autoimmune diseases (209, 210, 212). Several potential mechanisms have been proposed to improve inflammatory immune responses including lower NF-κB activity, increased PPAR-γ, reduced arachidonic acid-derived eicosanoids, intracellular T-cell signaling and membrane and lipid raft modification (210, 211). Overall, most evidence supporting the beneficial effects of long chain (n-3) PUFA on immune function is derived from studies conducted in acute or chronic severe inflammatory states in both animals and humans. Currently, very little is known about their potential efficacy in chronic low grade inflammatory states such as that which is seen in obesity. Refer to Table 1.2 for a summary of the immune modifying effects of EPA and DHA.

Table 1.2. Summary of the immune modifying effects of long chain (n-3) PUFA

IMMUNE FUNCTION	REFERENCE
Reduce the production of pro-inflammatory cytokines	(213-221)
Modify T-cell proliferative responses	(222-227)
Modify T-cell regulatory cytokines	(224)
Lower Th1 cytokine response	(226, 228)
Lower Th2 cytokine response	(229)
Modify antigen presentation	(230, 231)
Reduce inflammation-induced tissue damage	(232)
Improve Th1-driven disease	(219, 220, 232-238)
Improve Th2-driven disease	(229, 239, 240)

1.6.2.1 Human Intervention Studies

Based on upon the evidence in other inflammatory disease states, it would be logical for researchers to examine the impact of long chain (n-3) PUFA on the immune dysfunction associated with obesity. However, this is a newly emerging area of study and thus far, research has been limited to the effect of ALA or fish oil (FO) supplementation on systemic inflammation in overweight subjects. To date, there are no published reports on the effects of (n-3) PUFA on immune cell function in obese individuals. In general, there is more evidence to support a stronger anti-inflammatory and immune modulating role of EPA and DHA (210); however, the effects of ALA supplementation on circulating markers of inflammation in overweight subjects have been examined in three studies. Supplementation of ALA to obese and overweight subjects had a beneficial impact on systemic inflammatory markers in two studies (241, 242), but one study reported that flaxseed oil had no effect on several inflammatory markers in obese adults (243). Overall, it is difficult to state any definitive conclusions on the effectiveness of ALA in lowering systemic inflammatory markers and further work is required to determine if these effects are mediated via modification to immune cell function.

The effects of EPA and DHA on systemic inflammation in obesity has been examined to a greater extent that ALA, but is still very limited. Concentrations of serum inflammatory mediators (CRP, IL-6, sialic acid, soluble TNF receptors, plasminogen activator inhibitor-1 and TNF-α) were unaffected by supplementation of 1.1-4.2 g/d EPA and DHA for 6-12 wks in overweight or obese adults (244-247). One study did report that post-prandial monocyte chemoattractant protein-1 (MCP-1) was lower with fish oil supplementation (1.1 g/d EPA+DHA) compared to the placebo (high-oleic acid sunflower oil) group (247). Furthermore, Browning *et al* (2007 (248) postulated that the anti-inflammatory effects of EPA and DHA may be more effective in overweight or obese (BMI>25 kg/m^2) women with an inflammatory phenotype. Supplementation of 1.3g/d EPA and 2.9g/d DHA for 12 weeks lowered blood CRP and IL-6 concentrations relative to baseline (248). This study indicates that supplementation with EPA and DHA may be more effective in individuals with a heightened inflammatory phenotype.

In addition to pro-inflammatory mediators, the effects of long chain (n-3) PUFA on the anti-inflammatory adipokine, adiponectin, in overweight/obese adults has also been examined. While two separate groups have reported that 1.1 g/d EPA +DHA or 1.8 g/d EPA increased serum concentrations of adiponectin (245, 249), one reported an (n-3) PUFA-enriched diet (3.5% of energy, both ALA and EPA+DHA) for 14 weeks did not alter serum adiponectin levels in overweight and moderately obese adults (250).

In summary, investigations into the impact of dietary long chain (n-3) PUFA in overweight or obese individuals are limited to their effects on systemic markers of inflammation. They suggest that EPA and DHA have a minimal impact on pro- and anti-inflammatory mediators. Browning *et al*'s study does suggest that the anti-inflammatory benefits of long chain (n-3) PUFA may be more beneficial in overweight individuals with a greater inflammatory phenotype (248). However, further work is required to determine the appropriate doses of EPA, DHA and/or ALA in context of the dietary (n-6):(n-3) PUFA ratio. Furthermore, the underlying mechanisms and potential influence on immune cell function remain unexplored in the overweight population.

1.6.2.2 Animal Studies

Despite the vast literature in this area, there are few studies that have examined the impact of (n-3) PUFA on systemic inflammation and/or immune cell function in

obese/insulin-resistant rodents. Aguilera *et al* (251) explored the potential benefits of dietary (n-3) PUFA on a serum marker of inflammation in male Wistar rats given a sucrose solution (30% sucrose in water) for 21 weeks. After the induction of obesity, obese rats were assigned to a control diet (7.5% w/w fat; mixture of corn and canola oil, P:S=0.27, (n-6):(n-3)=9.3) or to the experimental fish oil diet (7.5% w/w fish oil, P:S=0.73, (n-6):(n-3)=0.02). The induction of obesity in these rats increased serum levels of TNF-α. However, similar to human supplementation studies feeding fish oil did not affect serum concentrations of this inflammatory cytokine compared to obese rats fed the control diet. Thus, (n-3) PUFA supplementation appears to have little impact on systemic inflammatory markers. However, other studies have reported that fish oil can beneficially modify the inflammatory environment of adipose tissue, including lower macrophage infiltration, and TNF-α concentrations as well as greater adiponectin levels (252, 253). In addition, both *ob/ob* mice and high-fat fed rodents supplemented with purified EPA or fish oil had higher circulating levels of adiponectin (249, 254, 255). Overall, similar to human studies, (n-3) PUFA have had little influence on systemic inflammatory markers such as TNF-α. However, adiponectin concentrations may be improved by supplementation and further work is necessary to determine the impact on immunity.

Guermouche *et al* (2004) (256) was the first to report the effects of fish oil supplementation on immune parameters in an obese rodent. Obese offspring of streptozotocin-induced gestational diabetic rats were provided either a control diet (5% vegetable oil, (n-6):(n-3) PUFA=28:1) or a diet enriched with DHA and EPA (2.5% vegetable oil and 2.5% fish oil, (n-6):(n-3) PUFA not determinable) (256). Relative to lean rats, splenocytes of obese offspring had impaired proliferative responses to ConA, which the authors postulate was due to higher intracellular Ca^{2+} concentrations. Feeding fish oil corrected the intracellular increases in Ca^{2+} and also normalized T-cell proliferative responses (256). This study supports that T-cell function is impaired in model of obesity and that feeding a diet rich in long chain (n-3) PUFA can correct these alterations. This paper is the first of its kind to explore the influence of EPA and DHA enriched diet on immune function in an obese animal model and points to a potential defect in T-cell function (i.e. calcium homeostasis). This research group has published one subsequent study regarding FO and immunity in obese rodents. In a similar study

design, macrosomic offspring fed FO (2.1%w/w, (n-6):(n-3) PUFA=0.5) had lower mRNA expression of IL-2 and IFN-γ and higher IL-4 in spleen tissue compared to obese rats fed containing 0.04%w/w (n-3) PUFA (or (n-6):(n-3) PUFA=26) (119). In addition, serum concentrations of IFN-γ were lower and concentrations of IL-4 were higher with fish oil supplementation (119). In general, feeding fish oil to young, obese rats lowered the Th1:Th2 cytokine balance in the spleen and serum (119).

Overall, (n-3) PUFA supplementation studies in obese rodents suggest that fish oil has modest influences on serum markers of inflammation. Moreover, T-cell function and the Th1:Th2 cytokine balance was improved by feeding a diet enriched with EPA and DHA. Although these findings are promising, these studies used very high levels of fish oil relative to the total dietary lipid. Approximately 50% of the 5%w/w dietary lipid was composed of (n-3) PUFA and this raises concern about the physiological relevance of such high levels in a very low fat diet. Furthermore, the very low level of (n-3) PUFA contained in the control diet may have also contributed to the impaired proliferative response of T-cells. Therefore, it is necessary to examine the effects of long chain (n-3) PUFA in the context of higher fat diets more relevant to the North American diet and to use a control diet with more sufficient levels of (n-3) PUFA. Currently, no one has assessed the effects of long chain (n-3) PUFA on immune cell phenotypes or inflammatory responses, nor have there been any reports of effects on gut-associated immunity in the obese state.

1.6.3 Mechanisms of PUFA Mediated Changes to Immune Cell Function

1.6.3.1 Membrane and Lipid Raft Composition

In addition to their role as a rich energy source, fatty acids are the major structural component of cell membranes. It is well established that the type and amount of dietary fat consumed influence the fatty acid composition of cell membrane phospholipids, including immune cells (257-259). In turn, manipulation of dietary fat has a profound effect on membrane structure and function (258). Modification of dietary PUFA can alter expression and function of ion channels (260), transporters (261), receptors (225) and membrane-bound enzymes (262) and signal transduction (263). The essential fatty acid composition of non-lymphatic cells has been reported to be altered in lean verse obese rodents (264-266), suggesting that altered phospholipid fatty acid composition may

contribute to altered immune responses in obesity. However, there are no published reports which link differences in membrane fatty acid composition with immune cell function in obesity.

Researchers have clearly established that increasing dietary (n-3) PUFA translates into greater cell membrane incorporation in many cell types, including immune cells, and in many disease states (257-259). Less is known regarding CLA and membrane fatty acid composition and function; however, studies have reported that CLA isomers are incorporated into immune cell phospholipids (267). Few researchers have reported isomer-specific changes in immune cells and any corresponding alterations in function. Furthermore, the influence of dietary CLA isomers on immune cell fatty acid composition has not been explored in an obese state. It is possible that the incorporation of CLA or (n-3) PUFA into membrane phospholipid fractions may differ in obese states due to the identified alterations in membrane essential fatty acid content.

A burgeoning area of membrane research involves specialized components of the membrane termed lipid rafts. These are cholesterol and sphinogmyelin rich microdomains which aggregate to facilitate protein interactions and signal transduction (268). Lipid rafts appear to be integral for immune cell function and investigators have verified the presence of lipid rafts in T-cells, B-cells, macrophages and dendritic cells (269-271). It appears that clustering of these membrane microdomains is essential for the formation of the immunological synapse (interface between T-cell receptor and the MHC molecule of antigen-presenting cells) and hence, required for adequate T-cell activation (268). Similar to the whole membrane, long chain (n-3) PUFA can modify the fatty acid composition of lipid rafts (272). Moreover, DHA has been shown to disrupt T-cell signalling, including decreased raft translocation of protein kinase C theta (PKC-θ), a key enzyme leading to transcription of IL-2 (263). Overall, evidence suggests that (n-3) PUFA mediate several aspects of immune cell function via modification of lipid rafts; however, it is unknown how they influence lipid raft composition in the obese state.

1.6.3.2 Gene transcription

In addition to membrane mediated effects, manipulation of dietary fatty acids can also directly or indirectly affect gene transcription (209, 273). Accumulating evidence indicates that PUFA can modulate several transcription factors involved in inflammatory

responses and T-cell proliferation (273). A feeding study conducted in healthy rodents revealed that long chain (n-3) PUFA can lower the activity of NF-κB and NFAT, transcription factors involved in activating IL-2 transcription (263). Furthermore, long chain n-3 PUFA and CLA are natural ligands of PPAR-γ and direct binding can lead to activation (274, 275).

The underlying mechanisms involved in obesity-induced immune abnormalities have been investigated to a very limited extent. It was suggested that lower proliferative response of T-cells in *fa/fa* Zucker rats was due to lower GLUT-1 expression, although a direct cause/effect relationship was not established (10). However, since this report (1998) there has been no further investigation into this proposed mechanism. Other groups have reported alterations in transcription factors that may be involved in impaired T-cell responses. Lamas *et al* (2003) (276) noted higher spleen (basal state) mRNA expression of PPAR-γ1 and lower DNA-binding activity of NF-κB. However, the author later failed to reproduce the PPAR-γ1 results in the same animal model (113). Thus, there is little support for the underlying biological mechanisms involved in the immune impairments of obesity. However, some of the above proposed mechanisms have been shown to be modified by long chain (n-3) PUFA and CLA.

1.7 SUMMARY

Obesity is a condition defined by a heightened inflammatory state and impaired immune responses. The existing literature is still somewhat conflicting and the underlying biological methods are poorly understood. Treatment of obesity includes dietary modification. As such, modification to dietary fat which may improve some of the metabolic disturbances could be easily incorporated into the treatment regimen of overweight subjects. Dietary polyunsaturated fatty acids, including CLA and (n-3) PUFA have shown beneficial effects in healthy individuals and in other inflammatory states. However, little is known about their potential efficacy to improve immune dysfunction in obesity.

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2 STUDY RATIONALE

2.1 RATIONALE

It has been postulated that the elevated inflammatory status observed in obesity is the major contributing factor to the onset and progression of associated chronic diseases, including cardiovascular disease, type 2 diabetes and cancer (1). This heightened inflammatory state is suggestive of altered adaptive immunity (or T cell activity) and there is indirect evidence to support this hypothesis from epidemiological studies. However, there have been few comprehensive immune studies conducted in overweight/obese humans or well defined animal models and direct support of this hypothesis does not exist. Furthermore, the underlying mechanisms involved in the specific impaired immune responses have not been defined. As the majority of the immune system is located outside of systemic circulation and immune function is affected by many environmental factors, animal models are required to initially define the effect of obesity on the adaptive immune response. Although there is some evidence suggesting that aspects of both the acquired and innate immune systems are impaired in rodent models of obesity, the results are not conclusive. This is likely due to the failure of researchers to clearly define the effects of obesity on immunity and the use of an undefined diet (rodent chow) that has no resemblance to the human diet, particularly in the content and composition of fat. It is important that careful consideration be used when designing rodent studies so that results can be extrapolated to the human population.

It is well established that changing the amount and composition of dietary fat influences both the innate and adaptive immune system in healthy animals and humans and in many chronic diseases. Despite the prevalence of obesity in our population, few have explored how dietary fat can influence immunity in the obese state. Research conducted in healthy rodents or models of acute inflammation have reported that CLA may beneficially alter T-cell function and the inflammatory response. Despite the early interest in the use of CLA to reduce adipose tissue mass in murine models of obesity, there have been no published reports regarding the effect of feeding CLA isomers on immunity in obese rodents. Only one study conducted in overweight adults with metabolic syndrome has examined the impact on immune cell function (2). This study reported that neither the c9t11 nor the t10c12 CLA isomers affected LPS-stimulated

cytokine production (2). However, the diet of these subjects was not measured or controlled for in this small (N=42, n=12-14/group) intervention and T-cell function was not assessed (2). Hence, the supportive data provided by studies in healthy rodents and humans and models of acute inflammation suggest that CLA isomers may be beneficial to the T-cell and inflammatory dysfunction in obesity. Furthermore, there is keen interest in marketing CLA as a potential, albeit questionable, treatment for obesity. Hence, it is relevant and important to establish the effects of feeding the major CLA isomers, c9t11 and t10c12, on immune cell function in obese rodents.

The long chain (n-3) PUFA, EPA and DHA, have garnered immense attention over the past few decades due to their immune modulating properties. Beneficial effects on chronic inflammatory conditions, including rheumatoid arthritis, have been reported in the literature (3). It is postulated that EPA and DHA exert their effects by modulating inflammatory T-cell responses (4). Thus, it is logical to predict that long chain (n-3) PUFA could beneficially modify immunity in the obese state. Additionally, feeding long chain (n-3) fatty acids has been demonstrated to be beneficial in the treatment of insulin resistance and hypertriglyceridemia (5), two metabolic abnormalities associated with obesity. There are few published reports that have examined immune cell function in obese subjects or rodent models. Human intervention studies have focused on systemic markers of inflammation and collectively they indicate that fish oil (FO) has a negligible impact in overweight or obese adults. On the other hand, animal supplementation studies suggest that FO can modify T-cell function, including down-regulating the Th1 response (6, 7). Although this offers support of the hypothesis, the diets used in these studies were very low in fat (5%w/w) and EPA and DHA accounted for nearly half of the fat composition (6, 7). Furthermore, the (n-6):(n-3) PUFA ratio in the control diet was very high (26:1) in comparison to a very low (n-6):(n-3) PUFA ratio (0.5) in the FO group. Thus, it is difficult to ascertain if the improvements in immunity observed in the FO group in this study were merely due to the extremely low level of (n-3) PUFA in the control diet. It is important to determine if these benefits persist when animals are fed diets in which the fat content and composition is designed to be more representative of the diet consumed by the population.

The mechanisms involved in dietary fatty acid mediated modification of immune cell function have been examined in cell culture systems as well as in healthy and disease rodent models. However, there has been little attempt to understand the molecular mechanisms involved in mediating immunity in obesity. One study has reported that FO may improve T-cell proliferative responses by modifying intracellular Ca^{2+} homeostasis (6). Based on studies conducted in healthy or disease states (8-10), dietary PUFA could alter immune cell function by modifying vital aspects of the cell membrane in obesity. More recently, lipid rafts have been identified as integral membrane components vital for cell-to-cell contact, receptor-ligand interaction and signal transduction (11) and long chain (n-3) PUFA have been shown to modify the fatty acid and protein composition (12). However, there are no reports to confirm that composition of lipid rafts are not altered in obesity. Dietary fat can also alter transcription factor activity and gene expression (13, 14). Overall, manipulation of dietary fatty acids can affect the function of cell membranes as well as transcription and activity of genes; however, this area also remains largely unexplored in the obese state.

Rodent models of obesity, consisting of both genetic and diet-induced, have allowed researchers to explore more specific aspects of immunity and to a greater extent than human studies. Impairments in T-cell function (15-17) and inflammatory cytokine production have been identified in obese rodents (18-22). However, there is no established model the best represents the immune dysfunction observed in human obesity. Therefore, it is necessary to describe and compare the accepted rodent models of obesity and insulin resistance using similar experimental conditions.

2.2 Objectives and Hypotheses

The overall purpose of this research was to determine the effects of obesity and dietary PUFA on immune function. The following specific objectives and hypotheses addressed this overall goal:

1) To establish the effect of obesity on immunity. It is hypothesized that:
    a. Obese (*fa/fa*) Zucker rats will have impaired T-cell function and greater inflammatory responses compared to lean Zucker rats.
    b. Obese JCR:LA-*cp* rats will have impaired T-cell and greater inflammatory cytokine responses compared to lean JCR:LA-*cp* rats.

c. T-cell and inflammatory cytokine responses of MLN immune cells will be altered in obese JCR:LA-*cp* rats.

d. High fat fed rats will have impaired T-cell and inflammatory cytokine responses.

2) To determine the effect of changing the composition of dietary fat on immune dysfunction in obesity. It was hypothesized that:

e. Dietary CLA isomers will be incorporated into immune cell phospholipids and will improve T-cell and inflammatory cytokine production in obese (*fa/fa*) Zucker rats.

f. Dietary long chain (n-3) PUFA will be incorporated into splenocyte membrane phospholipids and lipid rafts of obese JCR:LA-*cp* rats and will improve T-cell and inflammatory cytokine production in obese rats.

g. Dietary long chain (n-3) PUFA will be incorporated into MLN cell phospholipids and will improve stimulated T-cell and inflammatory cytokine production from MLN in obese JCR:LA-*cp* rats.

h. Dietary long chain (n-3) PUFA will be incorporated into splenocyte phosphatidylcholine and phosphatidylethanolamine and will modify T-cell and inflammatory cytokine production.

i. Dietary long chain (n-3) PUFA will modify T-cell stimulated IL-2 production by upregulating protein kinase C theta (PKC-θ).

3) To describe and compare the accepted rodent models of obesity and insulin resistance and determine which model best represents reports in human obesity. It is hypothesized that:

j. The immune responses in the high fat-fed obese rats will differ from that of *fa/fa* Zucker rats and JCR:LA-*cp* rats.

2.3 Chapter Format

The hypotheses stated above were tested in a series of experiments. These studies were organized in thesis chapters and have been prepared and/or submitted for scientific publication as individual manuscripts.

Chapter 3 reports the results of experiments that examined the effects of obesity and the major CLA isomers, combined and individually, on fatty acid incorporation into

splenocyte phospholipid membranes and immune cell function in lean and obese (*fa/fa*) Zucker rats. This chapter addresses objectives 1 and 2 and hypotheses (a) and (e).

Chapter 4 examines the effects of obesity and long chain (n-3) PUFA on the incorporation of fatty acids into splenocyte phospholipid membranes and lipid rafts. This study also investigates the impact of obesity and dietary long chain (n-3) PUFA on immune cell phenotypes and mitogen-stimulated cytokine production in JCR:LA-*cp* rats. Objectives 1 and 2 and hypotheses (b) and (f) are addressed in this chapter.

Chapter 5 examines the effects of obesity and long chain (n-3) PUFA on the fatty acid composition of MLN cell phospholipid membranes. This is the first study to investigate the impact of obesity and long chain (n-3) PUFA supplementation on MLN immune cell function. This chapter addresses objectives 1 and 2 and hypotheses (c) and (g).

Chapter 6 describes the effect of long-term feeding of long chain (n-3) PUFA on the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in obese JCR:LA-*cp* rats. Additionally, the impact of obesity and dietary long chain (n-3) PUFA on inflammatory cytokine production and T-cell function are examined, including the molecular mechanism leading to IL-2 production in ConA-stimulated splenocytes. This chapter addresses objectives 1 and 2 and hypotheses (b), (h) and (i).

Chapter 7 investigates the impact of high fat feeding on immunity under experimental conditions similar to those used for the genetic models. Comparisons are made among the three rodent models implemented in this research, including the *fa/fa* Zucker, JCR:LA-*cp* and high fed rats. This chapter attempts to distinguish a rodent model that most suitably represents the impaired immunity reported in human obesity. Objectives 1 and 3 and hypotheses (d) and (j) will be addressed in this chapter.

Chapter 8 summarizes the findings as they specifically pertain to the hypothesis and provides an overall general discussion.

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3 ABNORMAL IMMUNE RESPONSES IN *FA/FA* ZUCKER RATS AND EFFECTS OF FEEDING CONJUGATED LINOLEIC ACID[1,2]

3.1 INTRODUCTION

Obesity is associated with an increased risk of infection (as reviewed by (1)) and immune-related forms of cancer (2, 3), poor antibody responses to vaccines (4, 5) and increased levels of systemic (6) and tissue inflammatory mediators (7), indicating abnormalities in immune function. Although there is considerable evidence that chronic low grade inflammation is associated with the obese state, the aetiology of this inflammation is not known. Most studies have focused on the role of the inflammatory cells and the adipocyte in the aetiology of inflammation; however, T cells have an important role in regulating inflammation (8) and their contribution to inflammation in the obese state is not known.

The Zucker *fa/fa* rat is a monogeneic model of obesity that expresses a dysfunctional leptin receptor that severely limits its ability to respond to leptin (9), a condition that has been identified in only a few individuals (10). However, many of the metabolic abnormalities present in the Zucker *fa/fa* rat, including leptin resistance (as reviewed by (11)), are observed in human obesity (12, 13). A limited number of studies have been conducted on immune function in this animal model. Abnormalities in the innate immune system have been identified in the Zucker *fa/fa* rat including an impaired capacity to kill yeast, despite normal phagocytic function (14, 15). Additionally, there are several reports of T cell lymphopenia affecting both the $CD4^+$ and the $CD8^+$ T cells (16) and a decreased ability of lymphocytes to respond *in vitro* to mitogen stimulation (17, 18). Little is known about the effect of obesity on mitogen-stimulated cytokine, immunoglobulin production or immune cell types (beyond the relative proportion of $CD4^+$ and $CD8^+$ cells). Although these studies are suggestive of immune dysfunction, there is currently no animal model of obesity with identified chronic inflammation or clearly characterized T cell dysfunction that would explain the immune abnormalities

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[1] A version of this chapter has been published: Ruth MR, Taylor CG, Zahradka P and Field CJ. (2008) Abnormal Immune Responses in *fa/fa* Zucker Rats and Effects of Feeding Conjugated Linoleic Acid. *Obesity*; 16(8):1770-9.

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observed in human obesity. Therefore, the first objective of this study was to characterize immune function in the Zucker *fa/fa* rat to determine its suitability as a model for the inflammatory immune dysfunction associated with obesity in humans.

It is well established that dietary nutrients, particularly lipids, can influence both the inflammatory response and T cell function (19). More recently, conjugated linoleic acid (CLA), which describes a group of the geometric and positional isomers of the dietary essential linoleic acid, has been reported to have anti-inflammatory and immuno-regulatory effects in healthy animals (reviewed by (20)). Contrary to the animal studies, clinical trials in healthy individuals have reported few effects of CLA on immune function (21-27), including one report of a minor elevation in serum C-reactive protein (CRP) levels (28). Collectively, these results suggest that feeding CLA to healthy individuals has minimal impact on immune function. However, studies in animals suggest that CLA isomers may have a greater impact when the immune system is challenged. In support of this hypothesis, feeding a 1.3% w/w CLA mixture improved mucosal inflammation and increased mRNA levels of IL-10 in colonic lymph nodes in a piglet model of bacterial induced colitis (29). Furthermore, plasma levels of pro-inflammatory cytokines were lower and IL-10 levels were higher in animals with acute inflammation (immune mediated catabolism) fed a CLA mixture (1-2% w/w) (30, 31). The majority of studies that have been conducted in animals that have been fed a mixture of the two major isomers of CLA, the *cis*9,*trans*11 (c9t11) and *trans*10,*cis*12 (t10c12) CLA.

These findings suggest that feeding CLA isomers might be beneficial to treat the immune dysfunction associated with obesity. Contrary to what might be predicted from animal studies, two studies conducted in obese men reported minor elevations in blood C-reactive protein (t10c12 CLA only) and urinary 15-ketodihydroprostaglandin F2α with no effect on blood cytokine levels after 13 months of supplementation with either c9t11 or t10c12 CLA isomer (32, 33). It is well established for other dietary fats, such as the long chain (n-3) fatty acids, that the level and type of fats in the diet influence their ability to modulate inflammation (19). Unfortunately, in the study by Riserus *et al* (32, 33) the fat content and composition of the subject's diet was not determined.

To determine the potential benefits of CLA isomers in the treatment of obesity, systematic animal studies are required where the diet is controlled and the impact of the two main isomers are studied on the major immune abnormalities that occur with the obese state. The second objective of this study was to determine the effects of feeding c9t11 and t10c12 CLA, singly or combined, on parameters of immune function and inflammation in the *fa/fa* Zucker rat.

3.2 Materials and Methods

RPMI 1640 culture media, fetal calf serum, antimycotic-antibiotic solution (10,000 µg/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate and 25µg/mL amphotericin B), 2-mercaptoethanol and HEPES were purchased from Invitrogen (Burlington, ON, Canada). Phorbol myristate acetate (PMA) and Concanavalin A (ConA) were purchase from ICN (Montreal, PQ, Canada) and lipopolysaccarchide (LPS), ionomycin (I) and pokeweed mitogen (PWM) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). BD OptEIA anti-rat enzyme-linked immunoasorbant assay (ELISA) sets were used to detect interleukin (IL)-4, IL-6, IL-10, IFN-γ and TNF-α (BD Biosciences PharMingen, Mississauga, ON, Canada). IL-2 CytoSet was purchased from Biosource (Medicorp, Montreal, PQ, Canada). IL1-β ELISA kit was purchased from R&D Systems (Cedarlane Laboratories Ltd, Hornby, ON, Canada) and immunoglobulin (IgG, M and A) ELISA quantitation sets were purchased from Bethyl Laboratories Inc (Cederlane Laboratories, Ltd, Hornby, ON, Canada). Fluorescent pre-labelled monoclonal antibodies were purchased from BD Biosciences PharMingen (Mississauga, ON, Canada) except OX62 and OX12, which were purchased from Serotec (Cedarlane Laboratories Ltd, Hornby, ON, Canada). Streptavidin-Quantum Red™ was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Sterile 4mL tubes, 96-well "V"-bottom and flat bottom plates and 1.5mL microcentrifuge tubes were purchased from Fisher Scientific Company (Ottawa, ON, Canada). High performance thin layer chromatography plates were purchased from Fisher Scientific Company (Ottawa, ON, Canada) and glass methylation vials were purchased from Chromographic Specialties (Brockville, ON, Canada). All dietary components except cornstarch (Best Foods, Etobicoke, ON, Canada), CLA (Natural ASA, Hovdebygda, Norway) and tert-

butylhydroquinone (Aldrich Chemical Co, Milwaukee, WI) were purchased from Harlan Teklad (Madison, WI).

3.2.1 Animals and Diet

Experimental procedures were reviewed by the University of Manitoba, Fort Garry Protocol and Management committee and approved in accordance with the Canadian Council on Animal Care (CCAC) guidelines. Lean and obese male *fa/fa* Zucker rats (n=80, Harlan, Indianapolis, IN) were obtained at 5 wks of age and acclimatized for 5-7 days. Rats were fed a semipurified diet formula, based on the AIN-93G diet (34), differing only in the amounts of CLA isomers (Table 3.1). Lean and obese animals (n=10/treatment) were randomly assigned to one of the following dietary treatments for 8 wks: a) 0.4% w/w c9,t11 CLA (c9t11), b) 0.4% w/w t10,c12 CLA (t10c12) c) 0.4% w/w c9,t11 plus 0.4% w/w t10,c12 CLA (MIX), and d) 0% CLA (control diet, Ctl). The total amount of fat provided in the diet was 8.5% w/w. Our rational for providing the same concentration of the individual isomers in the mixture diet was to interpret the effects of the individual isomers and then their effect at the same concentration when provided together. The commercially prepared CLA isomers were in free fatty acid form. All dry ingredients were pre-mixed and fresh batches of diet containing oil were prepared weekly and stored at -20°C until fed.

The rats were individually housed in a temperature (21-23°C) and humidity (55%) controlled environment with 14:10 light to dark cycles. All rats had free access to water and were *ad libitum*-fed. Feed cups were filled 3 times per week and feed intake, adjusted for feed spillage, was recorded at that time. Animal body weights were recorded weekly. After consuming the experimental diets for 8 wks, rats were killed by CO_2 asphyxiation and cervical dislocation and the spleen removed.

Table 3.1. Diet composition

Ingredients	c9t11	t10c12	MIX	Ctl
	(g/kg of diet)			
Dry Mix				
Cornstarch	363	363	363	363
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Egg White	213	213	213	213
Cellulose	50	50	50	50
AIN-93 Mineral Mix	35	35	35	35
AIN-93 Vitamin Mix	10	10	10	10
Choline	2.5	2.5	2.5	2.5
Biotin Mix*	10	10	10	10
Tert-butylhydroquinone	0.014	0.014	0.014	0.014
Oil†				
Soy oil	81	81	76	85
c9t11 CLA	4.3	0	4.3	0
t10c12 CLA	0	4.3	4.3	0

* Biotin mix contains 200 mg biotin/kg of cornstarch

† - Total Oil in all diets= 85 grams/kg of diet. All dietary ingredients from Harlan Teklad (Madison, WI), except cornstarch (Best Foods, Etobicoke, ON) and CLA isomers Natural ASA (Hovdebygda, Norway)

3.2.2 Isolation of Splenocytes and Primary Culture Conditions

The spleens were weighed and placed in sterile 0.5% w/v bovine serum albumin in Krebs-Ringer HEPES buffer (pH 7.4) and isolated as we have previously described in detail (35). Isolated splenocytes were resuspended in complete culture media [RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic-antibiotic solution, HEPES (25 mmol/L), and 2-mercaptoethanol (2.5 mmol/L)] and counted on a haemacytometer (Fisher Scientific, Edmonton, AB, Canada). The fetal calf serum contains fatty acids and therefore the cell culture media contained 0.2μM c9,t11 CLA isomer. Splenocytes were resuspended in the culture media described above ($1.0x10^6$ cells/mL) and incubated in 4 mL sterile polystyrene tubes in a humidified atmosphere at 37°C in the presence of 5% v/v CO_2. The final cell culture medium either contained no mitogen (unstimulated cells) or was supplemented with mitogens at concentrations previously determined in our lab and/or published in the literature (ConA (2.5 mg/L) , LPS (1 m g/L) (36), PWM (55 mg/L), or PMA + I (20 μg/L + 0.5 nmol/L) (37) in healthy normal body weight rats. After 48 h of culture, the supernatant was

removed and stored at -80° C until cytokine, Ig and nitric oxide (NO) assays were performed. The cell pellets were washed with PBS and frozen at -80°C for fatty acid analysis.

3.2.3 Phenotype Analysis

In the control-fed rats, immune cell subsets in splenocytes were identified by one or two colour direct immunofluorescence assay as we have previously described (38). The following pre-labelled mAbs were used: CD3, RT1B (Class II monomorphic), CD28 (FITC-labelled); CD4, CD8, CD86, CD3, CD11b/c and OX12 (PE-labelled); and CD25, OX62, CD86 and CD80 (biotin-labelled). Streptavidin-Quantum Red™ (R-PE-Cy5 flurochrome) was added to wells containing biotin-labelled Ab. After final wash, plates were aspirated and 200 µL of cell fixative (1%w/v paraformaldehyde) was added to each well. The proportion of cells that were positive for each marker was determined by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA) according to the relative fluorescence intensity using CellQuest software (Becton-Dickinson).

3.2.4 Cytokine, Immunoglobulin and NO Production

The following assays were performed according to the manufacturer's instructions. The cultured cell supernatants of PMAI, ConA, LPS and PWM-stimulated splenocytes were used to determine IL-1β and TNF-α (31.2-2000pg/mL), IL-2 (23.4-1500 pg/mL), IL-4 (1.6-100 pg/mL), IL-6 (78-5000 pg/mL), IL-10 (15.6-1000 pg/mL) and IFN-γ (31.25-2000 pg/mL) levels with commercial ELISA kits (detection limit indicated in parentheses). Ig levels were quantified in LPS-stimulated and unstimulated (UNS) supernatant using ELISA quantitation kits. The range of detection for IgG, IgA and IgM were 7.8 ng/ml-500 ng/ml, 15-1000 ng/ml, and 31.2-2000 ng/ml, respectively. NO production was determined by analyzing nitrite (NO_2^-, a product of the L-arginine-dependent nitric oxide pathway) concentration in splenocyte culture supernatants using a colorimetric assay based on the Griess reaction (39). All samples were measured in duplicate and the absorbance was measured at 540 nm for NO_2^- or 450 nm for cytokines and Ig on a microtitre plate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA). The average of the duplicate data was used for statistical analysis if the coefficient of variance was ≤10%.

3.2.5 Splenocyte Phospholipid Fatty Acid Composition

A modified Folch method was used to extract lipids from freshly isolated splenocytes prior to mitogen stimulation as previously described (40). Total phospholipids were separated on silica-G plates as previously described (41) and visualized with 8-anilino-1-naphthalenesulfonic acid under ultraviolet light against the appropriate standards. Phospholipid fatty acid methyl esters were prepared from the scraped silica band. Methyl esters were prepared by the base-catalyzed method using sodium methoxide (NaOMe) as described elsewhere (42). Prepared phospholipid fatty acid methyl esters were flushed with nitrogen and stored at -35°C until analysis by gas chromatography. Fatty acids were separated by automated gas liquid chromatography (Varian 3800, Varian Instruments, Mississagua, ON) using a 100m *CP*-Sil 88 fused capillary column (Varian Inc) as described elsewhere (42).

3.2.6 Statistics

Statistical analysis was conducted using the SAS software statistical package (Version 9.1, SAS Institute, Cary, NC). All data was reported as mean ± SEM. The effects of diet and phenotype were determined by two-way ANOVA and significant differences between groups were identified by least square means at $p<0.05$. Blocking was imposed to account for any unexplained error associated with days animals were killed for cytokine, Ig and NO data. Phenotype analysis was conducted only on rats fed the control diet and a two-tailed t-test was used to compare differences between the phenotypes. Statistical significance was reported at $p \leq 0.05$.

3.3 RESULTS

Body weight, feed intake and spleen measurements:

Obese Zucker (*fa/fa*) rats consumed more feed, had significantly higher body and spleen weights and a lower spleen weight per gram (g) body weight, a lower number of total splenocytes and a lower number of splenocytes per g spleen weight than lean rats (Table 3.2). There was no effect of diet on any of the parameters in Table 3.2 in the lean animals. Obese rats fed the MIX or t10c12 CLA diets consumed significantly less feed than obese rats fed the Ctl diet and obese rats fed the t10c12 CLA diet consumed significantly less than the c9t11 and MIX CLA diets (Table 3.2). Obese rats fed the t10c12 and MIX diet had lower spleen weights than obese rats fed the Ctl or c9t11 diet (Table 3.2). Obese rats fed t10c12 and MIX diets had similar absolute spleen weights and number of splenocytes (t10c12 only) compared to lean rats fed the same diets.

Table 3.2. Effect of phenotype and CLA isomers on feed intake, body and spleen weight, and splenocyte numbers in lean and *fa/fa* Zucker rats

	Lean Rats				Obese Rats				Significance, p≤		
	Ctl	c9t11	t10c12	MIX	Ctl	c9t11	t10c12	MIX	Diet	Phenotype	Interaction
Feed Intake (g)	1022 ± 24	958 ± 21	999 ± 17	1023 ± 23	1665 ± 51*	1562 ± 55*‡	1399 ± 30†*	1539 ± 50*†‡	0.003	0.0001	0.008
Body Weight (g)	328 ± 5.3	336 ± 6.7	332 ± 8.8	326 ± 6.8	561 ± 13*	543 ± 13*	545 ± 9.8*	567 ± 18*	NS	0.0001	NS
Spleen Weight (g)	0.58 ± 0.01	0.56 ± 0.02	0.55 ± 0.02	0.55 ± 0.02	0.73 ± 0.05*	0.67 ± 0.03*	0.58 ± 0.02†	0.55 ± 0.04†	NS	0.0007	0.04
Spleen Weight per g Body Weight($x10^{-3}$)	1.8 ± 0.04	1.7 ± 0.06	1.7 ± 0.05	1.7 ± 0.03	1.3 ± 0.08*	1.2 ± 0.05*	1.1 ± 0.03*†	1.0 ± 0.05*†	0.0006	0.0001	NS
# of Splenocytes per Spleen ($1x10^7$)	22 ± 1.5	22 ± 1.2	21 ± 1.6	23 ± 1.4	20 ± 2.1	19 ± 1.6	20 ± 1.7	19 ± 2.0*	NS	0.007	NS
Splenocytes/g Spleen Weight ($1x10^8$)	3.9 ± 0.3	4.1 ± 0.3	3.7 ± 0.3	4.3 ± 0.2	2.8 ± 0.4*	2.8 ± 0.3*	3.4 ± 0.3	3.4 ± 0.3*	NS	0.0001	NS

Values are means±SEM, n=10. Significant effect of diet and phenotype as determined by two-way ANOVA. * Indicates different from lean group fed same diet in same row ($p \leq 0.05$); † Indicates different from obese ctl group in same row ($p \leq 0.05$); ‡ Indicates different from obese t10c12 group in same row ($p \leq 0.05$).

3.3.1 Pro-inflammatory Cytokines: TNF-α, IL-1β, IL-6 and IFN-γ

Splenocytes from obese Zucker rats produced more TNF-α, IL-1β and IL-6 than lean rats following mitogen stimulation (Table 3.3). There was no main effect of diet on the production of these cytokines by splenocytes from either lean or obese rats, regardless of the type of mitogen that was used. However, LPS-stimulated splenocytes of obese rats fed the t10c12 CLA diet produced less TNF-α and IL-1β than obese rats fed the Ctl diet ($p<0.05$, Table 3.3). Feeding CLA did not effect the production of TNF-α, IL-1β or IL-6 in lean rats. IFN-γ production in ConA stimulated splenocytes was higher in obese rats ($p<0.002$), but lower with PMAI ($p<0.0001$) and LPS ($p<0.0001$) stimulation. Lean animals fed the c9t11 CLA isomer produced less IFN-γ than lean animals fed the Ctl diet ($p<0.05$, PMAI-stimulated splenocytes). Diet did not affect IFN-γ production in ConA, LPS or PWM-stimulated immune cells of either lean or obese rats.

3.3.2 IL-2

Splenocytes from obese Zucker rats produced less ConA stimulated IL-2 ($p<0.0001$) than lean rats (Table 3.3), but there was no main effect of phenotype with PMAI or PWM stimulation (Table 3.3). Splenocytes of obese rats fed the c9t11 produced similar levels of IL-2 compared to lean rats fed the same diet (ConA, $p<0.05$); while splenocytes of obese rats fed the other diets had lower production compared to lean rats fed the same diet (ConA, $p<0.05$) (Table 3.3). Obese rats fed the MIX diet had decreased PMAI-stimulated production of IL-2 compared to obese rats fed the Ctl diet ($p<0.05$, Table 3.3). Feeding CLA did not affect PWM-stimulated IL-2 production in obese rats. Feeding either the MIX or the c9t11 diet to lean rats decreased IL-2 production in PWM-stimulated splenocytes ($p<0.05$). Lean rats fed any of three CLA diets produced less IL-2 compared to lean rats fed the Ctl diet (ConA, $p<0.05$) (Table 3.3).

3.3.3 IL-4 and IL-10

Immune cells from obese rats fed the c9t11 diet produced more IL-10 after ConA stimulation than lean rats fed the same diet and obese rats fed the MIX diet (Table 3.3). Obese rats fed the c9t11 CLA produced more IL-10 after stimulation with PMAI than cells from those fed the t10c12 and MIX CLA diets ($p<0.05$, Table 3.3). Obese rats fed the c9t11 diet produced more IL-10 after PWM stimulation compared to obese rats in the Ctl group ($p<0.05$, Table 3.3). Splenocytes of lean rats fed the c9t11 diet and stimulated

with PWM produced more IL-10 than lean rats fed the MIX diet. Diet or phenotype did not affect production of IL-10 in immune cells stimulated with LPS from lean and obese rats. Cells from obese rats fed any of the CLA containing diets produced less IL-4 than cells from Ctl-fed rats after stimulation with ConA or PMAI (Table 3.3). IL-4 production was not significantly different among the lean groups.

3.3.4 Ig and NO Production

Splenocytes from obese animals produced more IgA, IgG and NO in the unstimulated state and more IgM, IgA, IgG and NO after stimulation with LPS (Table 3.4). Feeding any of the CLA containing diets resulted in a lower ($p<0.05$) production of IgA both with and without LPS but only the amounts produced by the cells from the c9t11 and t10c12 fed animals reached levels not significantly different from lean animals fed the same diets (Table 3.4). Feeding the t10c12 or MIX diet to obese animals resulted in a lower production of NO in the unstimulated condition to levels not significantly different from the lean animals fed the same diets (Table 3.4). For lean rats, diet did not alter NO levels in LPS-stimulated or unstimulated cells.

3.3.5 Immune Cell Phenotypes in Spleen

Immune cell phenotypes were only measured for rats fed the control diet. Obese animals had a lower proportion of $CD3^+$ (42 ± 1.2 vs 49 ± 1.6, $p<0.004$), $CD3^+CD4^+$ (23 ± 1.2 vs 27 ± 1.8, $p<0.04$) and $CD8^+CD25^+$ (3 ± 0.5 vs 4 ± 0.5, $p<0.02$) cells and had a higher proportion of $CD11b/c^+$ (21 ± 1.5 vs 18 ± 1.4, $p<0.05$) and $OX6^+CD86^+$ (6 ± 0.8 vs 4 ± 0.8, $p<0.04$) cells in the spleen. There was no difference in the proportion of $CD3^+CD8^+$, $CD4^+CD25^+$, $CD4^+CD28^+$, $CD8^+CD28^+$, $OX12^+$, $OX6^+CD11b/c^+$, $OX6^+OX62^+$ and $OX6^+CD80^+$, and cells between lean and obese rats ($p<0.05$).

3.3.6 Phospholipid Fatty Acid Composition of Splenocytes

The relative proportions of fatty acids from 14:0 to 24:1 (n-9) in total phospholipids were measured but only major fatty acids are reported (Table 3.5). Obese animals had a significantly higher proportion of C14:0, C16:0, C18:1(n-9), C18:1(n-7), C20:3(n-6), C20:5(n-3), C22:5(n-3), and C22:6(n-3) and lower proportion of C18:2(n-6), C18:3(n-3), and C20:2(n-6) compared to lean rats. The phospholipids from obese rats had a higher proportion of total MUFA and (n-3) PUFA and a lower proportion of total (n-6) PUFA and (n-6):(n-3) PUFA ratio. Compared to the Ctl diet-fed rats, feeding any of the

CLA diets resulted in incorporation of the respective CLA isomer(s) into splenocyte phospholipids (Table 3.5). However there were significantly lower proportions of the individual CLA isomers in the phospholipids of obese rats compared to lean rats (Table 3.5). Obese rats fed the c9t11 CLA diet incorporated more C18:1(n-9), C18:1(n-7) and total MUFA and less C20:2(n-6) compared to obese rats fed the t10c12 or MIX diet ($p<0.05$). Obese rats fed the c9t11 di*et al*so had a higher proportion of C16:0 and a lower proportion of C20:3(n-6), C22:5(n-3) and (n-3) PUFA compared to obese rats fed the MIX diet ($p<0.05$).

Table 3.3. Effect of phenotype and CLA isomers on mitogen-stimulated cytokine production of splenocytes from lean and *fa/fa* Zucker rats.

		Lean				Obese				Significance, $p<$		
		Control	c9t11	t10c12	MIX	Control	c9t11	t10c12	MIX	Diet	Phenotype	Interaction
ConA	TNF-α	870 ± 202	680 ± 149	707 ± 156	669 ± 147	1160 ± 257	1308 ± 290*	1032 ± 228	1136 ± 267	NS	0.001	NS
	IFN-γ	1859 ± 326	1301 ± 304	1415 ± 314	1226 ± 300	2550 ± 697	2616 ± 582*	2065 ± 459	2272 ± 686	NS	0.002	NS
	IL-1β	369 ± 105	333 ± 59	330 ± 95	323 ± 122	648 ± 253	550 ± 233	358 ± 104	511 ± 160	NS	0.03	NS
	IL-2	1956 ± 307	1254 ± 238†	1266 ± 182†	1372 ± 191†	992 ± 136*	881 ± 125	731 ± 89*	635 ± 101*	0.0001	0.02	NS
	IL-4	24 ± 3.5	12 ± 2.7	12 ± 4.3	14 ± 0.8	16 ± 1.5	6.6 ± 0.8**	6.0 ± 1.8**	8.4 ± 0.4**	NS	NS	NS
	IL-6	2120 ± 498	1419 ± 332	1654 ± 368	1245 ± 276	2568 ± 603	2465 ± 549*	1884 ± 419	1907 ± 424	NS	0.004	NS
	IL-10	545 ± 120	530 ± 116	490 ± 107	497 ± 109	543 ± 119	704 ± 155*,***	542 ± 119	525 ± 115	NS	NS	NS
PMAI	TNF-α	758 ± 131	587 ± 103	652 ± 103	589 ± 130	944 ± 251	966 ± 204*	1021 ± 318	857 ± 179	NS	0.001	NS
	IFN-γ	2287 ± 286	1753 ± 318†	1970 ± 269	1888 ± 244	1414 ± 190	1370 ± 215	1210 ± 207*	1207 ± 299*	NS	0.0001	NS
	IL-2	4756 ± 284	4290 ± 362	4752 ± 252	4534 ± 283	4589 ± 281	4999 ± 192***	4594 ± 235***	3863 ± 242***	NS	NS	NS
	IL-4	43 ± 10	45 ± 9.0	41 ± 8.0	43 ± 10	55 ± 10	31 ± 5.0**	27 ± 6.0**	22 ± 5.0*,***	NS	NS	NS
	IL-6	1476 ± 341	1246 ± 203	1306 ± 278	1453 ± 323	2074 ± 493	2247 ± 617*	2013 ± 548*	1640 ± 365	NS	0.001	NS
	IL-10	534 ± 74	592 ± 78	522 ± 59	491 ± 108	543 ± 55	710 ± 115	469 ± 48†	431 ± 94†	0.02	NS	NS
PWM	TNF-α	605 ± 96	567 ± 108	437 ± 87	436 ± 72	1448 ± 244*	1369 ± 192*	1377 ± 224*	1432 ± 185*	NS	0.0001	NS
	IFN-γ	348 ± 80	338 ± 82	511±173	474 ± 113	426 ± 105	436 ± 192	320 ± 62	517 ± 144	NS	NS	NS
	IL-1β	309 ± 71	249 ± 59	259 ± 156	234 ± 55	518 ± 124*	495 ± 139*	478 ± 123*	553 ± 134*	NS	0.0001	NS
	IL-2	778 ± 297	479 ± 181**	612 ± 251	472 ± 153**	666 ± 308	531 ± 237	576 ± 211	574 ± 187	NS	NS	NS
	IL-6	1500 ± 296	1223 ± 296	1486 ± 264	1066 ± 139	1937 ± 350	1900 ± 360*	1694 ±293	1806 ± 389*	NS	0.0006	NS
	IL-10	742 ± 91	821 ± 125§	777 ± 77	599 ± 71	622 ± 66	875 ± 75**	710 ± 61	744 ± 57	NS	NS	NS

		Lean				Obese				Significance, $p<$		
		Control	c9t11	t10c12	MIX	Control	c9t11	t10c12	MIX	Diet	Phenotype	Interaction
LPS	TNF-α	530 ± 59	528 ± 90	491 ± 69	516 ± 54	1490 ± 126*	1309 ± 126*	1258 ± 155*,**	1339 ± 98*	NS	0.0001	NS
	IFN-γ	201 ± 55	325 ± 142	285 ± 106	366 ± 134	106 ± 23	90 ± 23*	99 ± 25	96 ± 17*	NS	0.0001	NS
	IL-1β	378 ± 59	341 ± 47	347 ± 49	312 ± 46	652 ± 101*	632 ± 93*	500 ± 57*,**	610 ± 106*	NS	0.0001	NS
	IL-6	3342 ± 301	3082 ± 501	3054 ± 543	3722 ± 406	4184 ± 320*	4541 ± 354*	4246 ± 315*	3962 ± 314	NS	0.0001	NS
	IL-10	846 ± 91	1065 ± 149	1017 ± 110	971 ± 128	1066 ± 94	1222 ± 94	996 ± 91	1078 ± 72	NS	NS	NS

Values are mean ± SEM, N=10. Significant effect of diet and phenotype as determined by two-way ANOVA. CLA, conjugated linoleic acid; ConA, concanavalin A; IFN-γ, interferon-γ; LPS, lipopolysacccharide; PWM, pokeweed mitogen; TNF-α, tumor necrosis factor-α; NS, not significant. * Indicates different from lean group fed same diet ($p<0.05$). ** Indicates different from Ctl group of the same phenotype ($p<0.05$); ***Indicates different from MIX group of same phenotype ($p<0.05$); † Indicates different from c9t11 group of same phenotype ($p<0.05$).

Table 3.4. Effect of phenotype and CLA isomers on IgM, IgA, IgG and NO production in unstimulated or LPS-stimulated splenocytes from lean and *fa/fa* Zucker rats.

		Lean				Obese				Significance, $p<$		
		Ctl	c9t11	t10c12	MIX	Ctl	c9t11	t10c12	MIX	Diet	Phenotype	Interaction
LPS	IgM	251 ± 43	383 ± 62†	347 ± 57†	330 ± 60	378 ± 62*	478 ± 51	477 ± 51	368 ± 45	NS	0.02	NS
	IgA	18 ± 4.3	25 ± 4.0	19 ± 4.2	15 ± 3.5	46 ± 6.5*	33 ± 3.3†	25 ± 3.6†	30 ± 4.0*†	0.04	0.0001	0.03
	IgG	37 ± 5.9	33 ± 3.5	33 ± 2.8	31 ± 3.3	53 ± 7.8*	48 ± 8.5*	38 ± 5.1†	36 ± 5.1†	NS	0.01	NS
	NO	7.4 ± 1.2	6.7 ± 1.1	7.7 ± 0.89	5.9 ± 0.93	8.9 ± 1.4	7.7 ± 0.87	9.1 ± 1.6	8.5 ± 1.3*	NS	0.007	NS
Unstimulated	IgM	407 ± 80	468 ± 91	406 ± 67	343 ± 72	402 ± 61	502 ± 60	433 ± 78	438 ± 71	NS	NS	NS
	IgA	21 ±2 .6	26 ± 3.8‡	19 ± 2.4	17 ± 2.3	37 ± 6.2*	31 ± 2.3†	24 ± 2.7†	31 ± 3.0*†	0.03	0.0001	NS
	IgG	29 ± 4.0	22 ± 2.1	23 ± 1.0	25 ± 2.9	40 ± 5.6*	36 ± 5.4*	32 ± 6.5	28 ± 3.1†	NS	0.003	NS
	NO	7.9 ± 1.2	6.7 ± 1.1	7.9 ± 1.2	7.7 ± 1.0	11 ± 1.4*	9.6 ± 1.1†	8.7 ±1.3†	8.2 ±1.1†	0.05	0.0007	NS

Values are mean ± SEM, N=10. Significant effect of diet and phenotype as determined by two-way ANOVA. *Indicates difference from lean rat fed same diet ($p \leq 0.05$); † Indicates difference from Ctl with the same phenotype ($p \leq 0.05$); ‡ Indicates different from t10c12 group of same phenotype ($p<0.05$).

Table 3.5. Effect of phenotype and CLA isomers on fatty acid composition of splenocyte phospholipids in lean and *fa/fa* Zucker rats.

	Lean Rats				Obese Rats				Significance, $p<$		
	Ctl	c9t11	t10c12	MIX	Ctl	c9t11	t10c12	MIX	Diet	Phenotype	Interaction
14:0	0.25 ± 0.02	0.31 ± 0.06	0.31 ± 0.02	0.30 ± 0.03	0.32 ± 0.02	0.36 ± 0.01	0.33 ± 0.02‡	0.42 ± 0.05*†	NS	0.006	NS
16:0	23 ± 0.9	23 ± 0.6	25 ± 0.6	24 ± 1.1	26 ± 0.9*	27 ± 0.7*‡	25 ± 0.8	24 ± 1.5	NS	0.01	NS
18:0	17 ± 0.8	17 ± 0.8	17 ± 0.8	17 ± 1.0	16 ± 0.5	16 ± 0.7	17 ± 0.6	15 ± 0.7	NS	NS	NS
18:1 (n-9)	7.9 ± 0.19	7.5 ± 0.09‡	7.1 ± 0.20†	6.8 ± 0.25†	7.8 ± 0.19	7.9 ± 0.23‡§	7.1 ± 0.13†	7.3 ± 0.28	NS	0.0006	NS
18:1 (n-7)	3.1 ± 0.07	3.1 ± 0.09	3.3 ± 0.15	3.0 ± 0.06	4.0 ± 0.12*	4.0 ± 0.11*‡§	3.6 ± 0.12†	3.6 ± 0.15*†	NS	0.0001	NS
18:2 (n-6)	11 ± 0.16	11 ± 0.23	12 ± 0.26	12 ± 0.59	7.6 ± 0.09*	7.8 ± 0.32*	8.4 ± 0.20*†	8.3 ± 0.31*	NS	0.0001	NS
18:3 (n-3)	0.53 ± 0.02	0.47 ± 0.01	0.42 ± 0.04	0.36 ± 0.09†	0.40 ± 0.02*	0.37 ± 0.02*	0.38 ± 0.01	0.37 ± 0.01	0.02	0.05	NS
c9t11 CLA	ND	0.28 ± 0.01†	ND	0.31 ± 0.01†	ND	0.20 ± 0.01*†	ND	0.21 ± 0.02*†	0.0001	0.0001	0.0001
t10c12 CLA	ND	ND	0.30 ± 0.02†	0.30 ± 0.01†	ND	ND	0.20 ± 0.02*†	0.20 ± 0.02*†	0.0001	0.0001	0.0002
20:2 (n-6)	1.3 ± 0.03	1.2 ± 0.02	1.2 ± 0.08	1.2 ± 0.08	0.63 ± 0.04*	0.59 ± 0.03*‡§	0.76 ± 0.04*†	0.73 ± 0.05*†	NS	0.0001	NS
20:3 (n-6)	1.1 ± 0.06	1.1 ± 0.04	0.86 ± 0.06	0.94 ± 0.05	1.5 ± 0.09*	1.3 ± 0.08‡	1.7 ± 0.09*	1.7 ± 0.13*	NS	0.0001	0.02
20:4 (n-6)	25 ± 0.7	27 ± 0.9	24 ± 0.5	26 ± 1.0	25 ± 0.7	25 ± 0.7	25 ± 0.7	26 ± 0.9	NS	NS	NS
20:5 (n-3)	0.21 ± 0.03	0.22 ± 0.01	0.19 ± 0.02	0.19 ± 0.02	0.33 ± 0.02*	0.31 ± 0.02	0.37 ± 0.03*	0.37 ± 0.03*	NS	0.0001	0.03
22:4 (n-6)	2.9 ± 0.15	2.7 ± 0.09	2.9 ± 0.24	3.0 ± 0.24	3.0 ± 0.15	2.6 ± 0.12	2.7 ± 0.14	3.0 ± 0.20	NS	NS	NS
22:5 (n-3)	1.4 ± 0.16	1.6 ± 0.11	1.6 ± 0.21	1.8 ± 0.17	2.3 ± 0.07*	2.0 ± 0.13‡	2.4 ± 0.20*	2.8 ± 0.21*†	0.03	0.0001	NS
22:6 (n-3)	1.4 ± 0.08	1.3 ± 0.05	1.4 ± 0.11	1.5 ± 0.16	1.8 ± 0.06*	1.5 ± 0.08	1.7 ± 0.09	1.8 ± 0.18	NS	0.002	NS

	Lean				Obese				Significance, $p<0.05$		
	Ctl	c9t11	t10c12	MIX	Ctl	c9t11	t10c12	MIX	Diet	Phenotype	Interaction
SFA	41 ± 1.2	41 ± 1.2	43 ± 0.5	41 ± 2.0	43 ± 1.3	43 ± 1.3	43 ± 1.3	41 ± 1.9	NS	NS	NS
PUFA	46 ± 1.3	45 ± 1.1	44 ± 0.9	46 ± 1.9	43 ± 1.0	41 ± 1.1	43 ± 1.2	44 ± 1.9	NS	0.02	NS
MUFA	12 ± 0.2	12 ± 0.2‡	12 ± 0.4	11 ± 0.3†	13 ± 0.4*	14 ± 0.4*‡§	12 ± 0.3†	13 ± 0.5*	0.006	0.0001	NS
PUFA:SFA	0.89 ± 0.05	0.91 ± 0.05	1.0 ± 0.03	0.91 ± 0.08	1.0 ± 0.05	1.1 ± 0.06	1.0 ± 0.06	1.0 ± 0.09	NS	NS	NS
(n-6) PUFA	43 ± 1.3	43 ± 1.0	41 ± 0.8	44 ± 1.8	40 ± 0.9	39 ± 1.1*	40 ± 1.2	42 ± 1.7	NS	0.01	NS
(n-3) PUFA	2.1 ± 0.05	2.0 ± 0.06	2.0 ± 0.16	2.1 ± 0.15	2.5 ± 0.08*	2.2 ± 0.09†‡	2.4 ± 0.10*	2.5 ± 0.21*	NS	0.0006	NS
(n-6):(n-3) PUFA	20 ± 0.6	21 ± 0.5	21 ± 2.4	22 ± 1.6	16 ± 0.5*	18 ± 0.8*†	17 ± 0.6*†	17 ± 1.1*	NS	0.0001	NS

Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids. Values are mean ± SEM, N=4(lean t10c12); N=5 (lean c9t11 and MIX); N=6 (lean Ctl); N=7(obese groups). Significant effect of diet and phenotype as determine by two-way ANOVA. * Indicates different from lean rat fed same diet ($p<0.05$); † Indicates different from Ctl with the same phenotype ($p<0.05$); ‡ Indicates different from MIX group of same phenotype ($p<0.05$); § Indicates different from t10c12 group of same phenotype ($p<0.05$).

a) Effects of Obesity on Immune Function		
T-cell Function		↓ IL-2 (ConA)
		↓ % Total T and T-helper cells
		↓ % Cytotoxic T-cells that express IL-2 receptor
		↑ IFN-γ (ConA)
		↓ IFN-γ (PMAI, LPS)
Inflammation		↑ IL-6 (LPS, PWM, PMAI, ConA)
		↑ TNF-α (LPS, PWM, PMAI, ConA)
		↑ IL-1β (LPS, PWM, ConA)
		↑ % macrophages
		↑ % activated antigen presenting cells
		↑ Igs & NO (LPS, unstimulated)
b) Effects of CLA on Immune Function in Obesity		
T-cell Function	c9t11	↑ IL-10 (ConA)
		similar IL-2 levels (ConA, c9t11 lean vs c9t11 ob)
Inflammation	c10t12	↓ IL-1β (LPS)
		↓ TNF-α (LPS)

Figure 3.1. Summary of the major biological findings on a) the effects of obesity on immune function in the Zucker *fa/fa* rat and b) on the effects of c9t11 or t10c12 CLA isomers on immune function in the Zucker *fa/fa* rat.

3.4 Discussion

3.4.1 Immune Dysfunction in the Obese *fa/fa* Zucker Rat

The results of this study demonstrate that the *fa/fa* Zucker rat, compared to its lean control, has altered immune function (refer to Figure 3.1). Other groups have also reported lower IL-2 production after mitogen stimulation in diet-induced obese rodents (36, 43) and a lower T-cell proliferative response (estimated by the rate of ^{3}H-thymidine incorporation) in both the *fa/fa* Zucker rat and diet-induced obese rats (16, 18, 36, 44). Consistent with findings in diet-induced obesity (16, 36, 44), *fa/fa* Zucker rats had a lower proportion and concentration of total T cells, affecting only the T helper ($CD3^{+}CD4^{+}$) subset, in the spleen. Unlike Tanaka *et al* (1998) (16) we did not see a lower proportion of cytotoxic T cells ($CD3^{+}CD8^{+}$) in the *fa/fa* Zucker. The lower number of total T cells would have contributed to the lower IL-2 production after stimulation with a polyclonal T cell mitogen, such as ConA (45, 46). Although the proportion of $CD8^{+}$ cells did not differ between lean and obese, obese rats also had a lower percentage of $CD8^{+}$ splenocytes that expressed the IL-2 receptor, suggesting that cytotoxic T-lymphocytes of obese rats may have a reduced capacity to proliferate.

Despite a lower production of IL-2, splenocytes isolated from *fa/fa* Zucker rats produced higher levels of inflammatory cytokines and NO (Figure 1). These results are novel and suggest a vigorous pro-inflammatory response by T cells and macrophages. It is possible that the slightly higher proportion of macrophages ($CD11b/c^{+}$) and activated antigen presenting cells ($RTB1^{+}$ or positive for major histocompatibility complex (MHC) II) that express a co-stimulatory molecule ($CD86^{+}$)) contributed to the higher production of these inflammatory mediators by cells from obese rodents. The small increase in the proportion of these innate immune cells is unlikely the sole contributor to the 2.5 – 2.8 fold increase in TNF-α production after LPS-stimulation. In agreement with previous reports, mitogen-stimulated production of the regulatory/anti-inflammatory cytokine, IL-10, did not differ between lean and obese rats (36). This pro-inflammatory response may be unique to the Zucker rat as there was no difference in TNF-α production by splenocytes after mitogen stimulation in diet-induced obese rodents (C57BL/6J mice or Wistar rats) (36, 43). Furthermore Lamas *et al* (2004) (47) reported that mRNA levels of TNF-α and IL-6 in spleen were actually lower in diet-induced obese rats. Alternatively, it

is possible that the highly saturated diets consumed by the rodents in these studies might have dampened the inflammatory reaction (48, 49). It is interesting that the *fa/fa* Zucker rat in the present study favored a pro-inflammatory response to a T cell mitogen while another study utilizing *ob/ob* mice reported lower inflammatory responses to allogeneic peripheral blood mononuclear cells or splenocytes (50). There is a growing body of evidence indicating that pro-inflammatory mediators can predict the onset of type 2 diabetes (51) and development of cardiovascular disease (52, 53) in humans. Thus, the heightened inflammatory responses we observed in obese animals may contribute to the disease pathology of obesity-associated co-morbidities.

To our knowledge, higher production of immunoglobulins in both the absence (increased IgA or IgG) and presence of LPS (increased IgG, IgA and IgM) has not been previously reported in obese animals. The preliminary analysis used to determine the optimal incubation time for cytokine and Ig production was conducted in lean rats and we acknowledge that the 48 h time point selected may not have been optimal as it was determined based on the maximum response in lean rats. Despite this, the heightened pro-inflammatory response observed in the obese animals, in the absence of a difference in the proportion of B-lymphocytes ($OX12^+$ cells) likely contributed to immunoglobulin production as elevated circulating levels of immunoglobulins have been reported in inflammatory conditions such as rheumatoid arthritis (54, 55).

It is well established that the type and amount of dietary fatty acids consumed influence the fatty acid composition of phospholipids in immune cells and this can modify membrane protein expression and function, membrane-mediated signalling and gene transcription (as reviewed by (56, 57)). Similar to previous studies that examined non-lymphatic tissues in the *fa/fa* Zucker rat, we observed abnormalities in the essential fatty acid concentration of splenocyte phospholipids (58-61). Consistent with the reported fatty acid composition of liver phospholipids in the *fa/fa* Zucker rat (58, 59), we observed a lower (n-6):(n-3) ratio in immune cell phospholipids. This was the result of both a higher proportion of total (n-3) fatty acids, including eicosapentaenoic acid (EPA, 20:5(n-3)), and a lower proportion of linoleic acid (18:2(n-6)) in the phospholipids of obese rats. It has been demonstrated in both human and animal feeding studies that lowering the (n-6):(n-3) ratio lowers the proliferative response of T-lymphocytes (62). Although this may

have contributed to the lower IL-2 response to ConA stimulation, it is inconsistent with the higher production of pro-inflammatory cytokines. Lowering the (n-6):(n-3) ratio is reported to reduce the inflammatory response of immune cells in both healthy and inflammatory states (as reviewed by (63)).

The underlying mechanisms responsible for the immune abnormalities reported in the *fa/fa* Zucker rat are unknown, though a few hypotheses exist. Although the current study was not designed to explore the underlying biological mechanisms, we propose that abnormalities in T-cell function may be related to the severe leptin resistance in this animal model. The Zucker *fa/fa* rat expresses a dysfunctional long form of the leptin receptor, which is present on B- and T-lymphocytes and monocytes/macrophages (64). When leptin was administered to ob/ob mice (leptin deficient mouse model) it improved T-lymphocyte responses to mitogens (increased IL-2 production and ^{3}H-thymidine incorporation) (50). The altered T-cell function we observed in the *fa/fa* Zucker rat might also be due to impairments in the protein kinase C (PKC) pathway due to leptin resistance. In the current study, splenocytes of obese rats stimulated with ConA, which directly binds to the T-cell receptor (65), produced more IFN-γ than lean rats. However, when splenocytes were stimulated with PMAI, which bypasses the plasma membrane receptors and activates PKC (66), less IFN-γ was produced by obese rats compared to lean rats. Leptin has been reported to stimulate the PKC pathway in peripheral blood mononuclear cells (67) and impairments in PMA stimulated PKC activity have been reported in *fa/fa* Zucker hepatocytes (68). Although leptin is reported to stimulate the production of inflammatory cytokines from macrophages (69) a leptin deficiency was protective against inflammatory experimental arthritis (70). Although we can not completely rule out an effect of leptin resistance, our results suggest that additional mechanisms contribute to the heightened inflammatory response we observed in splenocytes of *fa/fa* Zucker rats. Clearly, further investigation is warranted to determine the underlying biological mechanisms involved in the inflammatory immune dysfunction present in the obese state.

3.4.2 The Effect of Feeding Diets Containing CLA Isomers on Immune Function

Studies investigating the impact of CLA isomers on immune function in human obesity are extremely limited and are mostly restricted to non-specific markers of

inflammation (32, 33, 71). This is the first study to examine the effects of CLA on immune function in a rodent model of obesity and although we observed few changes these findings are important because CLA is marketed to the obese population for its weight reducing effects. Our results demonstrate that the individual CLA isomers modify some of the immune abnormalities in the obese *fa/fa* Zucker rat (Figure 1). Under the experimental conditions of the present study, we observed that feeding the c9t11 CLA isomer to obese rats may have a beneficial influence on the proliferative and immunoregulatory response of T-cells, whereas feeding the t10c12 CLA isomer reduced the inflammatory response after LPS-stimulation, while feeding both of these isomers together appeared to negate the immunological effects of the single isomers. Immune changes in the obese rodents can not be easily explained by the diet effects on feed intake (c9t11 only), body weight gain, or the distribution of T or B cells in spleen. It is unknown what effect the slight but significant decrease in feed intake (without a change in body weight) would have on immune parameters in obese rats fed the t10c12 or MIX CLA diet.

Dietary CLA has been reported to be incorporated into the phospholipid fraction of peripheral blood mononuclear cells (PBMC) in healthy humans (72). As expected, the CLA isomers were incorporated into splenocytes membranes in CLA-fed rodents but to a lower relative extent than in the obese animals (incorporation was 67-70% of lean rats). This is in agreement with a previous report from our group, which determined that CLA isomers are incorporated less into liver phospholipids of *fa/fa* rats compared to lean Zucker rats (73). Interestingly, despite greater incorporation into the phospholipid membrane of lean rats, CLA had little effect on the parameters of immune function measured in this study and does not explain the differences in immune responses between diets as splenocytes from rats fed MIX diet had similar levels of the two isomers as splenocytes from the groups fed the single isomer diets.

3.5 Conclusion

In conclusion, our results demonstrate that the *fa/fa* Zucker rat has T cell lymphopenia (mainly affecting the T helper subset) in the spleen and that this affects both T and B cell function. In addition to the lower incorporation of c9t11 or t10c12 CLA into the splenocyte phospholipids of obese rats, there was also a higher proportion of total

MUFA, (n-3) PUFA, a lower (n-6) PUFA and (n-6):(n-3) PUFA ratio and a lower proportion of linoleic acid. The pro-inflammatory response after stimulation is consistent with the inflammatory state of human obesity. A reduced ability to produce IL-2 after stimulation suggests a potential defect in T-cell function and is consistent with some of the immune abnormalities reported in obese humans. Feeding either the c9t11 (higher production of IL-10) or t10c12 (lower production of TNF-α and IL-1β) isomers singly but not together modulated the inflammatory response and proliferative response of splenocytes when stimulated. Further research is needed in obese humans to determine the physiological importance of these changes.

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4 FEEDING LONG CHAIN (N-3) POLYUNSATURATED FATTY ACIDS TO OBESE LEPTIN RECEPTOR DEFICIENT JCR:LA-*CP* RATS MODIFIES IMMUNE FUNCTION AND LIPID RAFT FATTY ACID COMPOSITION[1,2]

4.1 INTRODUCTION

It is well established that higher levels of circulating biomarkers of inflammation are present in the obese state, strongly suggesting inappropriate immune activation or regulation (as reviewed by(1)). In addition, obese individuals are more likely to develop other chronic inflammatory conditions, including certain forms of cancer (2, 3), cardiovascular disease (CVD) and type 2 diabetes and specific markers of inflammation can predict the development of CVD (4, 5) and type 2 diabetes (6). Impairments in the acquired immune system have also been identified in the overweight population. Individuals with a higher BMI are reported to be at an increased risk of infection and infection-related mortality (as reviewed by (7)), have poor antibody responses to vaccines (8-10) and immune cells have a reduced capacity to proliferate when stimulated with T-cell mitogens (11, 12). Overall, these studies support that immune function is abnormal in obesity, although the literature in humans is sparse. It is generally concluded that dysregulation of inflammatory responses is the key link among metabolic syndrome, cardiovascular disease and type 2 diabetes.

The JCR:LA-*cp* rat is a genetic model of obesity that expresses a dysfunctional leptin receptor which prevents any known receptor (ObR) mediated signal of leptin. Rats that are homozygous for the autosomal recessive *cp* gene (*cp*/*cp*) are obese and insulin resistant, have dyslipidemia and develop atherosclerosis (13-15). Consequently, this model has been used extensively to study the impact of metabolic syndrome on various parameters of CVD; however, this is the first study to examine immune function. Several researchers have demonstrated that overweight humans also have higher circulating levels of inflammatory mediators (as reviewed by (1)). Although one group has noted impairments in the proliferative response to T-cell mitogens (11, 12), the contribution of

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[1] A version of this chapter has been accepted for publication. "Feeding long chain (n-3) polyunsaturated fatty acids to obese leptin receptor deficient JCR:LA-*cp* rats modifies immune function and lipid raft fatty acid composition" Megan R. Ruth, Spencer D. Proctor & Catherine J. Field. (2008) Br J Nutr.

[2] Content of this chapter was presented at the International Society for the Study of Fatty Acids and Lipids, Cairns, AU, July 2008.

T-cells to inflammation or the effect of inflammation on T-cell function in the obese state is unknown.

It is well established that dietary nutrients, particularly lipids, can influence T cell function and the inflammatory response (16). Of particular interest in the literature have been the (n-3) PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have potent effects on immunity and inflammation that improve chronic inflammatory conditions (as reviewed by (17)). However, the impact of feeding fish oil to obese rodents on immune parameters is limited to only three studies, including one that reported no effect of fish oil on serum TNF-α concentrations (18). One research group, using macrosomic offspring of diabetic rat dams, reported that feeding EPA and DHA improved the proliferative response of splenocytes to ConA and lowered the Th1:Th2 of serum cytokines (19, 20). These studies indicate that feeding fish oil to obese offspring lowers the Th1 inflammatory response and improves T-cell proliferative response; however, there is little support for EPA and DHA's role in more specific aspects of the innate immune system in obesity.

Although the immune modifying potential of EPA and DHA has been examined in various chronic inflammatory states, there is very little evidence for their effects on immune health in overweight adults. Collectively, these studies indicate that supplementing mixtures of EPA and DHA (1.1-4.2g/d) for a short duration (6-12wk) have only a limited effect on systemic markers of inflammation in obese men or women, but offer little insight into the direct impact of long chain (n-3) PUFA on immune cell function (21-24).

An emerging area of interest in immunology is the role that lipid rafts have on immune cell signalling and general function. Lipid rafts are membrane microdomains found within the plane of the plasma membrane. They are enriched in cholesterol and sphingolipid and are insoluble in non-ionic detergents at low temperatures (25). Lipid rafts serve as platforms for the aggregation of specific membrane-bound components requiring coordinated assembly for signal transduction (as reviewed by (26)). Recently it was concluded that lipid rafts may be partly responsible for the (n-3) PUFA-mediated effects on immune cells (as reviewed by (27)); however, these studies are limited to *in vitro* experiments or studies involving healthy mice.

The objectives of this study were to determine if immune function was altered in obese JCR:LA-*cp* rats fed a high (n-6) PUFA diet and to determine if feeding a diet supplemented with long chain (n-3) polyunsaturated fatty acids would 1) improve the immune dysfunction present in the JCR:LA-*cp* rat and 2) alter the fatty acid composition of lipid rafts in immune cells of obese rats. Furthermore, we chose to examine two levels of EPA and DHA; the lower level to reflect what could be easily achieved through supplements and dietary modifications and the higher level to represent therapeutic levels, which would require considerable manipulation of the food supply and greater supplementation.

4.2 Materials and Methods

4.2.1 Animals and Diet

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Agricultural, Life and Environmental Sciences Animal Ethics Committee at the University of Alberta. Male obese (*cp/cp*) and lean (+/+ or +/?) rats of the JCR:LA-*cp* strain were raised in our breeding colony at the University of Alberta. All animals were individually housed in a temperature and humidity controlled environment with a 12/12-h reversed light cycle. Animals were weaned at 3 weeks of age and given free access to water and standard laboratory rat non-purified diet (Lab diet 5001, PMI Nutrition International, Brentwood, MO, USA). At 12 weeks of age 1%w/w cholesterol (Sigma-Aldrich, Oakville, ON, Canada) was added to the rat non-purified diet of all rodents for 2 wks to accelerate the atherosclerotic disease process in the JCR:LA-*cp* rodents (28). At 14wks, *cp/cp* rats were randomly allocated to receive one of the following nutritionally complete diets (n=10/diet) for 3 wks: control (0% EPA+DHA, 1% w/w cholesterol), low fish oil (LFO, 0.8% w/w or 1.7% of calories EPA+DHA, 1%w/w cholesterol) or high fish oil (HFO, 1.4%w/w or 3.1% of calories EPA+DHA, 1% w/w cholesterol); lean (+/+ or +/?) rats (n=5) were allocated to the control diet for 3 wks. A 3 week feeding period was chosen based on a previous dietary intervention study in the JCR:LA-*cp* (29). The nutrient composition of the experimental diets is provided in Table 4.1 and the fatty acid composition of the fat included in these diets is provided in Table 4.2. Fresh batches of diet containing oil were prepared weekly and stored at 4°C until fed, feed cups were

replaced every 2-3 days to ensure that the lipid did not oxidize. Rats were weighed and feed consumption, adjusted for food spillage, was recorded twice weekly. After an overnight fast, rats were killed by cardiac exsanguination under iso-fluorane anaesthesia. Blood was collected via cardiac puncture in BD Vacuntainer® (BD Biosciences, Mississauga, ON, Canada) and serum stored at -80°C until later analysis. The spleen was removed under aseptic conditions.

Table 4.1. Composition of experimental diets.

	Ctl	LFO	HFO
Diet Ingredient		g/kg	
Casein (high protein)*	267	267	267
Corn Starch¶	212	212	212
Dextrose *	215	215	215
Non-nutritive cellulose*	79	79	79
Fat Mixture	148	148	148
AOAC Vitamin Mix *	9.4	9.4	9.4
Bernhart-Tomerelli Mineral Mix *	48	48	48
Choline Bitartrate*	2.7	2.7	2.7
Inositol *	6.2	6.2	6.2
L-methionine*	2.5	2.5	2.5
Cholesterol‡	10	10	10
Fat Mixture		g/kg	
Flaxseed Oil§	3	3	3
Stearine††	91	91	94
Sunflower Oil†	54	40	24
Fish Oil **	0	14	27

*Harlan-Teklad (Madison, WI); † Save-On Foods (Edmonton, AB, Canada); ‡ Sigma (Oakville, Canada); § Holistic® Flaxseed oil (London Drugs, Edmonton AB, Canada); ††Completely hydrogenated soybean oil (CanAmera, Edmonton, AB, Canada); ¶Safeway (Edmonton, AB, Canada); **Fish Oil contained 3mg/g mixture of alpha tocopherols, (Ocean Nutrition Dartmouth, NS, Canada)

Table 4.2. Fatty acid composition of experimental diets.

	Control	LFO	HFO
	g/100g fatty acids		
14:0	0.08	0.14	0.13
16:0	8.51	10.56	7.60
18:0	54.42	53.65	55.97
18:1 (n-9)	9.29	8.42	5.52
18:2 (n-6)	23.40	17.11	16.59
18:3 (n-3)	1.25	1.57	1.22
20:5 (n-3)	0.00	3.67	6.35
22:5 (n-3)	0.00	0.02	0.01
22:6 (n-3)	0.00	1.67	3.00
Total PUFA	25	24	28
Total SFA	65	66	65
PUFA:SFA	0.4	0.4	0.4
Total (n-6) PUFA	24	18	17
Total (n-3) PUFA	1.3	7.0	10.7
(n-6):(n-3) PUFA	19	3	2
Total EPA+DHA	0.0	5.4	9.4

Abbreviations used: SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

4.2.2 Isolation of Splenocytes and Primary Culture Conditions

The spleens were weighed and placed in sterile 0.5% w/v bovine serum albumin in Krebs-Ringer HEPES buffer (pH 7.4) and splenocytes were isolated as we have previously described in detail (30). Isolated splenocytes were resuspended in complete culture media (RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic-antibiotic solution, HEPES (25 mmol/L), and 2-mercaptoethanol (2.5 mmol/L)) and counted on a hemacytometer (Fisher Scientific, Edmonton, AB, Canada). The essential fatty acid composition (w/w) of the fetal calf serum was: 4.4% 18:2 (n-6); 0.1% 18:3(n-3); 0.5% 20:2(n-6); 3% 20:4(n-6); 0.1% 20:5(n-3); 0.1% 22:4(n-6); 0.3% 22:5(n-3); and 0.8% 22:6(n-3). Splenocytes (1.25×10^6 cells/L) were resuspended in the culture media described above and incubated in 4 mL sterile polystyrene tubes in a humidified atmosphere at 37°C in the presence of 5% v/v CO_2. Splenocytes were cultured without mitogen (unstimulated cells) or with ConA (2.5mg/L), LPS (1mg/L) or PWM (55mg/L) as we have previously described (31). After 48 h of culture, the

supernatant was removed and stored at -80° C until cytokine assays were performed. Preliminary studies confirmed that by 48 h the maximum production of cytokines was achieved for cells from both lean and obese rats (data not illustrated).

4.2.3 Phenotype Analysis

Immune cell subsets in freshly isolated splenocytes were identified by one, two or three colour direct immunofluorescence assay as we have previously described (32). The following pre-labelled mAbs were used: CD3, OX6 and CD28 (FITC-labelled); CD4, CD8, CD3, CD11b/c and OX12 (PE-labelled); and CD25, and CD8 (biotin-labelled). All antibodies were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada) except CD4 and OX12, which were purchased from Serotec (Cedarlane Laboratories Ltd, Hornby, ON, Canada) and Streptavidin-Quantum Red™ was purchased from Sigma-Aldrich (Oakville, ON, Canada). Streptavidin-Quantum Red™ (R-PE-Cy5 flurochrome) was added to wells containing biotin-labelled Ab. After final wash, plates were aspirated and 200 uL of cell fix (1% w/v paraformaldehyde) was added to each well. The proportion of cells that were positive for each marker was determined by flow cytometry (FACScan; BD Biosciences, Mississauga, ON, Canada) according to the relative fluorescence intensity using CellQuest software (BD Biosciences, Mississauga, ON, Canada).

4.2.4 Cytokine Production and Serum Haptoglobin

All of the following assays were performed according to the manufacturer's instructions. The cultured cell supernatants of unstimulated, ConA, LPS and PWM-stimulated splenocytes were used to determine IL-1β and TNF-α (detection limit: 31.2-2000pg/mL), IL-2 (23.4-1500 pg/mL), IL-4 (1.6-100 pg/mL), IL-6 (78-5000 pg/mL), IL-10 (15.6-1000 pg/mL) and IFN-γ (31.25-2000 pg/mL) levels with OptEIA anti-rat enzyme-linked immunoasorbant assay (ELISA) sets were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada). Values below the range of detection were assigned half of the value of the lowest standard value. Serum haptoglobin levels were determined by a colorimetric assay purchased from Tri-Delta Development Limited (Maynooth, Ireland) according to the manufacturer's instructions. All samples were measured in duplicate and the absorbance for cytokine assays was measured at 450nm and 630nm for haptoglobin on a microtitre plate reader (SPECTRAmax 190,

Molecular Devices, Sunnyvale, CA). If the coefficient of variance exceeded 10% for duplicate samples, the samples were re-run. The average of the duplicate data with a coefficient of variance of ≤10% was used for statistical analysis.

4.2.5 Lipid Raft Isolation

Lipid rafts were isolated from unstimulated, freshly isolated splenocytes from obese rats only, as previously described by our group (33). Briefly, $2x10^8$ of freshly isolated splenocytes were lysed in 500µl of TNE (25 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA) containing 1% (v/v) Triton X-100 (VWR) with fresh protease and phosphatase inhibitors (Sigma-Aldrich) for 30min at 4°C. Lysates were spun for 30min at 4°C (1000rpm) and the supernatant was transferred to cooled ultracentrifuge tubes (Beckham Coulter, Mississauga, ON, Canada). An 80% w/v (800g/L in TNE) sucrose solution was added to the supernatant to make a 40% w/v sucrose solution. The lysates were gently overlaid with 2ml of a 30% w/v (300g/L in TNE) sucrose solution, followed by 2mL of a 5% w/v (50g/L in TNE) sucrose solution on ice. Samples were centrifuged for 8 h at 268,000g at 4°C in an Optima Max Ultracentrifuge MLS-50 rotor (Beckham Coulter). Based on previous experiments described below, the lipid raft material (1mL) was collected from the 5/30% glucose interface. Ice-cold TNE solution was added to raft fractions centrifuged at 268,000 g for 30 min to pellet and concentrate the rafts.

Initial experiments were conducted using 4 obese JCR:LA-*cp* rats that were fed the control diet to confirm the isolation of lipid rafts. Six consecutive 800µl samples were taken starting at the top of the gradient and the fractions were stored at -80°C until dot-blotting was performed. A bicinchoninic acid assay (Sigma-Aldrich) was used to determine the protein concentration in each raft fraction. Each fraction (2µg/10µl) was dot-blotted onto a nitrocellulose membrane and antibodies directed against the positive raft marker, GM1-ganglioside (cholera toxin B subunit conjugated with horseradish peroxidase (Sigma) and the negative raft marker, transferrin receptor (Zymogen Laboratories, Invitrogen, Burlington, ON, Canada) were used to confirm the isolation of lipid rafts. The GM1-ganglioside marker stained most strongly in the third fraction and the transferrin receptor stained most intensely in fraction 4-6 and least intensely in fraction 1-3 (Figure 4.1).

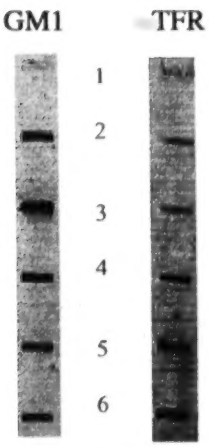

Figure 4.1. Immunoblots characterizing lipid rafts isolated from obese JCR:LA-*cp* rats fed control diet. GM1, positive raft marker; TFR (transferrin receptor), negative raft marker. Numbers represent fraction number, where 1 is the top layer and six is the bottom layer. Fraction 3 represents raft fraction and 4-6 represent soluble membrane factions.

4.2.6 Splenocyte Phospholipid Fatty Acid Composition

A modified Folch method was used to extract lipids from freshly isolated, unstimulated splenocytes and lipid rafts (fraction 3) as previously described (34). For lipid extracted from whole cells, total phospholipids were separated on silica G plates as previously described (35) and visualized with 8-anilino-1-naphthalenesulfonic acid under ultraviolet light against the appropriate standards. FA methyl esters were prepared from splenocyte total phospholipids and from total lipid of lipid rafts (34) and were separated by automated gas liquid chromatography (Varian 3800, Varian Instruments, Mississagua, ON) using a 100m *CP*-Sil 88 fused capillary column (Varian Instruments) as described elsewhere (36).

4.2.7 Statistics

Statistical analysis was conducted by one way ANOVA using the SAS software statistical package (Version 9.1, SAS Institute, Cary, NC, USA). All data were reported as mean ± standard error of the mean (SEM). Significant differences among groups were determined by Duncan's multiple range test ($p<0.05$) and all non-parametric data were log-transformed prior to running statistical analyses. Differences between groups with data that remained nonparametric after log-transforming were analyzed using Kruskal-Wallis/Wilcoxon test ($p<0.05$).

4.3 RESULTS

4.3.1 Feed Intake, Body weight and Spleen Characteristics

Obese rats had higher daily feed intake (33 ± 0.4 g/d vs 20 ± 0.3 g/d, $p<0.05$), final bodyweight (592 ± 5 g vs 378 ± 4 g, $p<0.05$), and spleen weight (1165 ± 40 mg vs 856 ± 23 mg, $p<0.05$) than lean rats. When adjusted for body weight (bwt), spleens of obese rats weighed less than lean rats (1.98 ± 0.06 mg/g bwt vs. 2.27 ± 0.06 mg/g bwt, $p<0.05$), but the concentration of splenocytes per gram of spleen did not differ between lean and obese rats (5.3 ± 0.5x10^8 cells vs. 5.6 ± 0.4x10^8 cells, $p<0.05$). Obese rats fed LFO (556 ± 16 g) or HFO (561 ± 6 g) diet had lower final body weights than the obese rats fed the control diet (592 ± 5 g, $p<0.05$), but change in body weight (76 ± 11 g, Ctl vs. 85 ± 4 g, LFO vs 80 ± 5 g, HFO) and feed intake did not differ (34 ± 0.6, LFO or 32 ± 0.3, HFO). Feeding FO did not alter spleen weight (LFO, 1930 ± 130mg, or HFO, 1910 ± 75mg) or concentration of immune cells (LFO, 5.2 ± 0.6x10^8, or HFO, 5.7 ± 1.0x10^8) in the spleen.

4.3.2 Fatty Acid Composition of Splenocyte Phospholipids

Obese rats fed the control diet had a higher proportion of C16:0, C18:1(n-9), C20:3(n-3), C22:5(n-3), C22:6(n-3) and total MUFA and (n-3) PUFA and a lower proportion of C18:0, C24:1(n-9) and (n-6):(n-3) PUFA ratio in splenocyte phospholipids, compared to the lean animals fed the same diet (Table 4.3). Obese animals fed either FO diet had a significantly higher proportion of C18:1(n-9), C20:5(n-3), 22:5(n-3) and total (n-3) PUFA and lower proportion of C20:4(n-6), C24:1(n-9) and a lower (n-6):(n-3) PUFA ratio. Only rats fed the LFO diet had a significantly higher proportion of 18:2(n-6), compared to the obese rats fed the control diet. Compared to the HFO diet, rats fed the LFO diet had higher percentage of C20:4(n-3) and (n-6):(n-3) PUFA and a lower percentage of 20:5(n-3) and total (n-3) PUFA. Feeding fish oil did not significantly change the proportion of C22:6(n-3) that was incorporated into the splenocyte phospholipid membrane of the obese JCR:LA-*cp* rat (Table 4.3).

Table 4.3. Fatty acid composition of splenocyte phospholipids in lean rats fed the Ctl diet and obese rats fed the Ctl, LFO or HFO diet.

	Lean Ctl	Obese Ctl	LFO	HFO
	(g/100g)			
14:0	0.31 ± 0.09	0.36 ± 0.04	0.39 ± 0.04	0.41 ± 0.03
16:0	23 ± 1^{a}	26 ± 1^{b}	26 ± 1^{b}	26 ± 1^{b}
18:0	30 ± 1^{a}	23 ± 1^{b}	23 ± 0.78^{b}	22 ± 1^{b}
18:1 (n-9)	6.9 ± 0.3^{b}	7.6 ± 0.6^{b}	8.4 ± 0.2^{a}	8.9 ± 0.1^{a}
18:1 (n-7)	1.6 ± 0.01^{b}	2.8 ± 0.04^{a}	2.9 ± 0.09 ^{a}	3.0 ± 0.10^{a}
18:2 (n-6)	8.1 ± 0.7^{b}	8.0 ± 0.4^{b}	9.4 ± 0.3^{b}	9.0 ± 0.3^{ab}
18:3 (n-3)	0.38 ± 0.04^{b}	0.43 ± 0.01^{ab}	0.49 ± 0.02^{a}	0.44 ± 0.02^{ab}
20:3 (n-6)	0.86 ± 0.17	0.61 ± 0.10	0.58 ± 0.07	0.63 ± 0.10
20:4 (n-3)	0.6 ± 0.1^{c}	1.4 ± 0.1^{ab}	1.5 ± 0.1^{b}	1.2 ± 0.1^{b}
22:1 (n-9)	0.20 ± 0.06^{a}	0.43 ±0.07^{ab}	0.31 ± 0.05^{b}	0.26 ± 0.05^{b}
20:4 (n-6)	20 ± 2^{a}	18 ± 1^{a}	13 ± 1^{b}	11 ± 0^{b}
20:5 (n-3)	0.1 ± 0.0^{c}	0.7 ± 0.2^{c}	2.1 ± 0.2^{b}	3.5 ± 0.1^{a}
24:0	0.88 ± 0.18	0.85 ± 0.12	0.80 ± 0.12	0.94 ± 0.14
22:3 (n-3)	0.70 ± 0.10	0.75 ± 0.15	0.82 ± 0.14	0.93 ± 0.14
24:1 (n-9)	2.4 ± 0.2^{a}	1.3 ± 0.0^{b}	0.58 ± 0.05^{c}	0.42 ± 0.04^{c}
22:4 (n-6)	0.26 ± 0.02^{a}	0.23 ± 0.02^{ab}	0.23 ± 0.04^{ab}	0.16 ± 0.02^{b}
22:5 (n-3)	0.85 ± 0.08^{c}	2.2 ± 0.3^{b}	3.5 ± 0.4^{a}	4.4 ± 0.7^{a}
22:6 (n-3)	1.0 ± 0.1^{b}	2.3 ± 0.2^{a}	2.6 ± 0.3^{a}	2.9 ± 0.3^{a}
MUFA	12 ± 0^{b}	13 ± 0^{a}	13 ± 0^{a}	14 ± 0^{a}
SFA	55 ± 2	51 ± 2	51 ± 1	50 ± 1
PUFA	34 ± 3	35 ± 2	34 ± 2	35 ± 1
PUFA:SFA	0.61 ± 0.07	0.70 ± 0.05	0.67 ± 0.05	0.71 ± 0.03
PUFA (n-6)	31 ± 3^{a}	30 ± 1^{ab}	27 ± 1^{b}	26 ± 1^{b}
PUFA (n-3)	2.7 ±0.1^{d}	5.5 ± 0.4^{c}	7.5 ± 0.6^{b}	9.4 ± 0.4^{a}
(n-6):(n-3)	11 ± 1.3^{a}	5.5 ± 0.3^{b}	3.7 ± 0.2^{c}	2.8 ± 0.1^{c}

Data represent mean ± SEM; n=10/group for obese rats and n=5/group for lean rats. Means within the same row that do not share a common letter are significantly different ($p<0.05$). The relative proportions of fatty acids from 12:0 to 24:1(n-9) were measured in whole splenocyte membrane, but only the major fatty acids were reported. Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

4.3.3 Fatty Acid Composition of Lipid Rafts

Obese rats fed either FO diet had a higher proportion of 18:2(n-6), 20:5(n-3), 22:5(n-3) and total (n-3) PUFA and a lower proportion of 20:4(n-6) and (n-6):(n-3) PUFA ratio (Table 4.4). Only rats fed the HFO diet had a higher proportion of 16:1 (n-9), 18:1 (n-9), and 22:6(n-3). Compared to rats fed the HFO diet, rats fed the LFO had a higher proportion of 18:0, and a lower percentage of C18:1*cis*11, C20:5(n-3), total MUFA, total (n-3) PUFA and a lower (n-6):(n-3) PUFA ratio (Table 4.4).

Table 4.4. Splenocyte fatty acid composition of total lipid from lipid rafts of obese JCR:LA-*cp* rats fed the Ctl, LFO or HFO diet.

	Obese Ctl	LFO	HFO
		g/100g	
14:0	0.79 ± 0.04	0.83 ± 0.07	0.94 ± 0.08
16:0	37 ± 1	37 ±	37 ± 1
18:0	27 ± 1^{ab}	29 ± 1^{a}	26 ± 1^{b}
18:1 (n-9)	5.1 ± 0.1^{b}	5.7 ± 0.4^{b}	6.9 ± 0.4^{a}
18:1 (n-7)	2.2 ± 0.1^{ab}	2.1 ± 0.1^{b}	2.4 ± 0.1^{a}
18:2 (n-6)	3.7 ± 0.2^{b}	4.5 ± 0.3^{a}	4.5 ± 0.3^{a}
18:3 (n-3)	0.35 ± 0.01	0.37 ± 0.02	0.38 ± 0.02
20:3 (n-6)	0.68 ± 0.05	0.62 ± 0.06	0.60 ± 0.06
20:4 (n-3)	1.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
20:4 (n-6)	7.0 ± 0.4^{a}	4.9 ± 0.4^{b}	4.6 ± 0.4^{b}
20:5 (n-3)	0.17 ± 0.02^{c}	0.64 ± 0.06^{b}	1.3 ± 0.15^{a}
24:1	2.6 ± 0.2	2.3 ± 0.2	2.6 ± 0.2
22:4 (n-6)	2.1 ± 0.2	1.7 ± 0.2	2.1 ± 0.2
22:5 (n-3)	0.82 ± 0.10^{c}	1.3 ± 0.14^{b}	1.9 ± 0.20^{a}
22:6 (n-3)	0.68 ± 0.06^{b}	0.69 ± 0.10^{b}	1.0 ± 0.12^{a}
MUFA	12 ± 1^{ab}	12 ± 1^{b}	14 ± 1^{a}
SFA	67 ± 1	69 ± 2	65 ± 2
PUFA	17 ± 1	16 ± 1	18 ± 1
PUFA:SFA	0.26 ± 0.02	0.24 ± 0.02	0.28 ± 0.03
(n-6) PUFA	14 ± 1	12 ± 1	13 ± 1
(n-3) PUFA	3.4 ± 0.2^{c}	4.5 ± 0.3^{b}	5.9 ± 0.5^{a}
(n-6):(n-3) PUFA	4.2 ± 0.2^{a}	2.7 ± 0.1^{b}	2.1 ± 0.1^{c}

Data represent mean ± SEM (N=9/group). Means within the same row that do not share a common letter are significantly different ($p<0.05$). The relative proportions of fatty acids from 12:0 to 24:1(n-9) were measured, but only the major fatty acids were reported. Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

4.3.4 Phenotype of Splenocytes

Obese rats fed the control diet had a higher proportion of $CD3^+CD4^+$ and $CD11b/c^+OX6^-$, but a lower proportion of $CD4^+CD25^+$, $CD11b/c^+OX6^+$, and $OX12^+$ (B-cells) splenocytes compared to lean rats fed the control diet (Table 4.5). Obese rats fed FO had a higher proportion of $CD3^+$, $CD3^+CD4^+$, $CD4^+CD28^+$, $CD4^+CD28^-$ and $CD11b/c^+OX6^-$ splenocytes, but only those rats fed the LFO diet had a higher proportion of $CD4^+CD25^+$ splenocytes and only rats fed the HFO diet had a lower proportion of $CD11b/c^+OX6^+$ (Table 4.5).

Table 4.5. Splenocyte phenotypes of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA rats fed the Ctl, LFO or HFO diet.

	Lean Ctl	Obese Ctl	LFO	HFO
$CD3^+$	46 ± 1^{ab}	44 ± 1^{b}	48 ± 1^{a}	49 ± 2^{a}
$CD4^+$	37 ± 0^{c}	42 ± 1^{b}	46 ± 1^{ab}	46 ± 1^{a}
$CD3^+CD4^+$	33 ± 0^{b}	33 ± 1^{b}	38 ± 1^{a}	37 ± 2^{a}
$CD8^+$	16 ± 1	15 ± 1	15 ± 0	15 ± 0
$CD3^+CD8^+$	13 ± 1	13 ± 2	13 ± 0	12 ± 1
$CD4^+CD25^+$	8.8 ± 0.5^{a}	5.9 ± 0.8^{b}	9.1 ± 0.5^{a}	6.9 ± 0.6^{ab}
$CD4^+CD25^-$	44 ± 2.0^{a}	41 ± 0.91^{ab}	39 ± 1.0^{b}	40 ± 1.1^{b}
$CD8^+CD25^+$	3.6 ± 0.5	2.8 ± 0.5	3.5 ± 0.4	2.4 ± 0.5
$CD8^+CD25^-$	17 ± 1^{a}	15 ± 1^{ab}	13 ± 1^{b}	15 ± 1^{ab}
$CD4^+CD28^+$	NM	44 ± 1^{b}	50 ± 1^{a}	48 ± 1^{a}
$CD8^+CD28^+$	NM	11 ± 1	11 ± 1	9.3 ± 0.2
$CD4^+CD28^-$	NM	6.3 ± 0.4^{a}	4.4 ± 0.4^{b}	4.8 ± 0.3^{b}
$CD8^+CD28^-$	NM	4.4 ± 0.3	4.7 ± 0.6	4.2 ± 0.4
$CD11b/c^+OX6^+$	11 ± 0.46^{a}	6.7 ± 0.9^{b}	6.6 ± 0.6^{b}	4.0 ± 0.35^{c}
$CD11b/c^+OX6^-$	4.2 ± 0.4^{c}	5.8 ± 0.4^{b}	7.2 ± 0.5^{a}	7.4 ± 0.4^{a}
$CD11b/c^+$	12 ± 1^{ab}	12 ± 1^{ab}	14 ± 1^{a}	11 ± 1^{b}
$OX6^+$	38 ± 3^{a}	36 ± 1^{ab}	32 ± 2^{b}	31 ± 1^{b}
$OX12^+$	42 ± 2^{a}	37 ± 1^{b}	35 ± 1^{b}	36 ± 1^{b}

Data represent mean ± SEM; n=10/group for obese rats and n=5/group for lean rats. Values are a proportion of the total gated cells as determined by immunofluoresence. Means within the same row that do not share a common letter are significantly different ($p<0.05$). NM = not measured.

4.3.5 Cytokine Production

Splenocytes of obese rats fed the control diet produced less mitogen-stimulated IFN-γ (ConA, LPS and PWM), less LPS-stimulated IL-1β, and less ConA-stimulated IL-10 compared to lean rats fed the control diet (Fig. 4.2-4.4). Unstimulated splenocytes of

obese rats fed the control diet produced less IL-6 (26 ± 4 pg/ml vs. 61 ± 20 pg/ml) and IL-10 (348 ± 23 pg/ml vs. 499 ± 86 pg/ml) than lean rats fed the control diet. IL-2, IL-4 or IL-10 (LPS or PWM-stimulated) (Fig. 4.2-4.4) or unstimulated TNF-α or IL-1β (data not shown) production did not differ between lean and obese rats.

Feeding either FO diet to obese rats resulted in lower production of IL-1β (LPS or PWM-stimulated), IL-10 (PWM-stimulated), IFN-γ (PWM and ConA-stimulated) and IL-4 (ConA-stimulated) compared to obese rats fed the control diet (Fig. 4.2-4.4). Obese rats fed the LFO diet had lower LPS-stimulated IFN-γ production and lower IL-10 (236 ± 54 pg/ml vs. 348 ± 23 pg/ml) production by unstimulated splenocytes. Obese rats fed the HFO diet had higher ConA-stimulated IL-10 production compared to obese control rats and lower LPS-stimulated IL-1β compared obese rats fed the LFO. Feeding FO to obese rats did not alter IL-2 or IL-6 (Fig. 4.2-4.4) production or unstimulated TNF-α or IL-1β (data not shown).

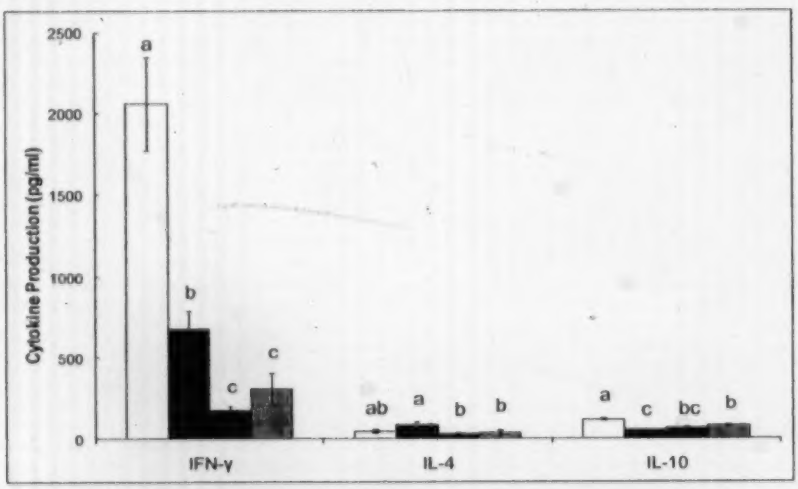

Figure 4.2. ConA-stimulated splenocyte cytokine production of lean and obese JCR:LA-*cp* rats fed the Ctl diet and obese rats fed LFO or HFO diets. Bars represent mean ± SEM (n=10/group for Ob Ctl, LFO and HFO and n=5 for Ln Ctl). Bars not sharing a common letter are significantly different ($p<0.05$). □ Lean Ctl; ■ obese Ctl; ■ LFO; ■ HFO. ConA-stimulated IL-2 (1328 ± 79, n=34); TNF-α (62 ± 7, n=35; IL-6 (180 ± 12, n=35) were not statistically different among groups.

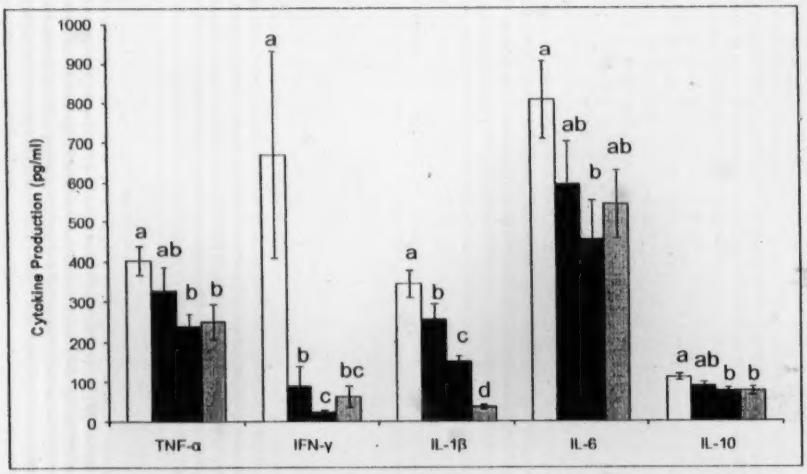

Figure 4.3. LPS-stimulated splenocyte cytokine production of lean and obese JCR:LA-*cp* rats fed the Ctl Diet and obese rats fed FO. Bars represent mean ± SEM (n=10/group for Ob Ctl, LFO and HFO and n=5 for Ln Ctl). Bars not sharing a common letter are significantly different ($p<0.05$). □ Lean Ctl; ■ obese Ctl; ■ LFO; ■ HFO.

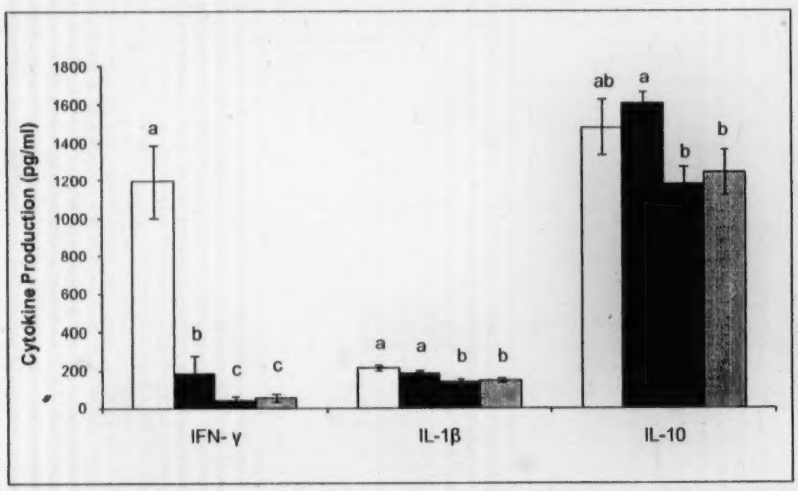

Figure 4.4. PWM mitogen-stimulated splenocyte cytokine production of lean and obese JCR:LA-*cp* rats fed the Ctl Diet and obese rats fed FO. Bars represent mean ± SEM (n=10/group for obese Ctl, LFO and HFO and n=5 for lean Ctl). Bars not sharing a common letter are significantly different ($p<0.05$). □ Lean Ctl, ■ obese Ctl, ■ LFO, ■ HFO. PWM-stimulated TNF-α (273 ± 22, n=35); IL-2 (184 ± 15, n=34); IL-6 (289 ± 19, n=34) were not statistically different among groups.

4.3.6 Haptoglobin

The concentration of serum haptoglobin in either the LFO-fed rats (1.6 ± 0.1 mg/ml) or HFO-fed rats (1.6 ± 0.1 mg/ml) did not differ from obese Ctl rats (1.7 ± 0.1 mg/ml) ($P<0.05$).

4.4 DISCUSSION

4.4.1 Immune Dysfunction in the JCR:LA-*cp* Rat

This preliminary study demonstrated altered immune responses in the JCR:LA-*cp* rat, an established model of the metabolic syndrome. In comparison to lean rats, obese rats had a lower inflammatory response (defined by IL-6 production) in the unstimulated condition and lower production of inflammatory cytokines (IL-1β and IFN-γ) with mitogen stimulation. The lower production of LPS-stimulated IFN-γ is consistent with our previous findings in Zucker *fa/fa* rats (31), a study in *ob/ob* mice lymph nodes (37) and a study using diet-induced obese (DIO) mice (38). However, ConA-stimulated IFN-γ production was also lower in obese rats. We and other groups have reported higher T-cell mitogen induced IFN-γ production in splenocytes of *fa/fa* Zucker rats (31). Similar to our observation in the current study that unstimulated splenocytes of obese rats produced less IL-6, Lamas *et al* (2004) reported lower IL-6 mRNA levels in basal state splenocytes of DIO rats (39). In obese humans, the effects of obesity on *ex vivo* mitogen-stimulated cytokine production are limited to two studies. Although one study has reported that blood mononuclear cells of obese individuals produce more TNF-α in response to LPS (12), a study conducted in morbidly obese patients reported significantly less production of monocyte chemoattractant protein-1 (MCP-1) and IFN-γ in response to LPS and PMAI (phorbol 12-myristate 13-acetate + ionomycin) stimulation (40). This study suggests that inflammatory cytokine production in the JCR:LA-*cp* rat may better represent morbid obesity in humans. However, despite the lower (n-6):(n-3) PUFA ratio in membranes and a leptin receptor defect, IL-2 production (a measure of T-cell proliferative response) after mitogen stimulation was not different between obese and lean JCR:LA-*cp* rats suggesting that unlike Zucker *fa/fa* rats and genetic and diet-induced rodent models of obesity (31, 41, 42, 42-46) mitogen induced proliferation by splenocytes does not appear to be impaired in obese JCR:LA-*cp* rats. Currently, mitogen-stimulated IL-2 production in obese individuals has not been measured *ex vivo*. However, 2 groups

have reported that there was no difference in the percentage of PMAI-stimulated $CD4^+$ cells that expressed IL-2 in obese children (47, 48). This suggests that the JCR:LA-*cp* rat represents the mitogen-stimulated production of IL-2 observed in human obesity.

4.4.2 Leptin Receptor and Immunity

The JCR:LA-*cp* rat expresses a dysfunctional form of the leptin receptor due to a single point mutation in the corpulent (*cp*) or leptin receptor gene (49, 50). As a consequence, the extracellular domain of the leptin receptor is absent and thus obese (*cp/cp*) rats lack the ability to respond to leptin via the long form of the leptin receptor (Ob-Rb). Recent evidence indicates that leptin has a significant role in immune cell function (37, 51-56) and is expressed on several immune cell types, including macrophages and dendritic cells and T- and B-lymphocytes (54, 57). Researchers have identified leptin as a key regulator of the inflammatory response of both the innate and acquired immune systems (37, 51-56). Therefore, the absence of a functional leptin receptor signalling via the Ob-Rb likely accounts, at least in part, for the impaired production of inflammatory mediators in splenocytes of the JCR:LA-*cp* rat (58). Our finding that ConA-stimulated IL-2 production is unaltered in obese JCR:LA-*cp* rats is surprising in light of evidence that suggests leptin is critical for the proliferative response of $CD4^+$ T-lymphocytes, prevention of T-lymphocyte apoptosis and secretion of IL-2 (as reviewed by (59)). This suggests that leptin is not critical for adequate IL-2 secretion, a measure of T-cell proliferation in mitogen-stimulated splenocytes.

4.4.3 Effect of Feeding Fish Oil on Immune Function in Obese JCR:LA-*cp* Rats

Feeding fish oil reduced the production of inflammatory cytokines by splenocytes in this rodent model, without altering the proliferative response (measured by IL-2 production) of T-lymphocytes. It is interesting that despite normal IL-2 production, feeding EPA and DHA lowered both the Th1 (IFN-γ) and Th2 (IL-4) cytokines when JCR:LA-*cp* rat splenocytes were stimulated with a T-cell mitogen. It is difficult to ascertain if this finding is specific to this rodent model or reflective of the obese state, as few studies have examined the impact of fish oil on immune function in obese and/or insulin resistant conditions. While we did not include a group of lean rats fed fish oil, previous studies in non-obese rodents have shown that dietary (n-3) PUFA suppress IL-2 production (60-63). It has been recently postulated that (n-3) PUFA lower both the Th1

and Th2 cytokine responses in other inflammatory disease states, which is consistent with our data (as reviewed by (16)).

In addition to the effects on the adaptive immune system, feeding fish oil also resulted in a lower innate immune response as determined by *ex vivo* LPS-stimulated cytokine production. Feeding the higher level of EPA and DHA resulted in a significantly lower LPS-stimulated IL-1β production and higher ConA-stimulated IL-10 suggesting that the higher dose is more effective at suppressing the inflammatory response than the lower dose. It is well established in other inflammatory disease states that high doses of EPA and DHA can reduce the inflammatory response and improve disease pathology (as reviewed by (17)). Consistent with studies feeding FO to overweight subjects (21-24), the (n-3) PUFA diets did not influence a serum marker of inflammation (haptoglobin). The HFO diet contained slightly higher levels of total monounsaturated fatty acids compared to the Ctl diet. Changes in the monounsaturated fatty acid content of the diet have been reported to alter immune function, but the differences in these studies far exceeded the 3% difference in the present study (64, 65).

4.4.4 Effects of Diet and Obesity on Lipid Membrane Composition

The differences in PUFA composition of the phospholipid membrane between lean and obese rats may have contributed to the lower production of inflammatory cytokines. Obese rats fed the control diet had a lower ratio (n-6):(n-3) PUFA ratio due to a higher incorporation of total (n-3) fatty acids, including docosahexaenoic acid (DHA, 22:6(n-3)). The lower phospholipid (n-6):(n-3) PUFA ratio agrees with our previous findings (31) and others using the *fa/fa* Zucker rat (66, 67). It is well established that lowering the (n-6):(n-3) PUFA ratio in the membrane of immune cells can reduce the inflammatory response in both healthy and inflammatory states (as reviewed by (17)). Obese JCR:LA-*cp* rats had significant differences in the fatty acid composition of splenocyte phospholipids despite consuming the same diet as lean rats (Table 3), suggesting an abnormality in fatty acid metabolism.

Feeding fish oil further lowered (n-6):(n-3) PUFA ratio in splenocyte membrane obese of rats, due to both a higher content of EPA and DPA (docosapentaenoic acid, 22:5(n-3)) and lower AA. Recently it has been suggested that PUFA-mediated alterations in immune cell function can be explained, in part, by changes in the protein and lipid

content and composition of lipid rafts (as reviewed by (68)). In the current study, compared to obese rats fed the control diet, obese rats fed FO had significantly higher incorporation of linoleic acid, EPA and DPA and a lower proportion of AA and (n-6):(n-3) PUFA in lipid rafts isolated from splenocyte. Only obese rats fed the HFO diet had a significantly higher percentage of DHA in the lipid component of splenocyte rafts. Our results suggest that there is a preferential incorporation of (n-3) PUFA into the lipid raft domains comparative to the whole membrane when FO is fed. Relative to the whole membrane, there was a greater increase of EPA into the lipid raft when FO was fed (76% and 265% greater for the LFO and HFO diets, respectively). Furthermore, in relation to the whole membrane, incorporation of DHA into the lipid raft was 21% greater for rats fed the higher dose of fish oil. The significantly higher incorporation of DHA into the lipid raft of obese rats fed the HFO diet may have contributed to the finding that JCR:LA-*cp* rats fed the HFO diet had a lower proportion of activated innate immune cells ($CD11b/c^{+}OX6^{+}$, non-B-cells expressing the major histocompatibility complex (MHC) class II molecule). The MHC class II molecule has been shown to reside in membrane microdomains of dendritic cells and macrophages/monocytes (69-72) and (n-3) fatty acids can down-regulate the expression of MHC II molecules on immune cells from healthy rodents (73, 74). Thus, it is possible that the significantly higher proportion of DHA in splenocyte lipid rafts of rats fed the HFO diet could have displaced the MHC Class II molecule from the lipid raft component of the splenocyte membrane and contributed to the lower inflammatory response (lower LPS-stimulated IL-1β).

4.5 Conclusion

This is the first study to report that the JCR:LA-*cp* rat, a genetic rodent model of obesity and insulin resistance, has impaired immune responses. With the exception of IL-2 production, splenocytes of obese rats were poor responders to mitogen stimulation. There was lower mitogen-stimulated inflammatory cytokine production in these rats which may be due to the higher proportion of (n-3) polyunsaturated fatty acids in splenocyte phospholipid membranes. Feeding fish oil to obese rats reduced mitogen-stimulated inflammatory cytokine production without affecting ConA-stimulated IL-2 production, possibly via modification to the fatty acid composition of the whole membrane and lipid raft. Furthermore, the high fish oil diet improved the inflammatory

response to a greater extent than the lower fish oil diet (lower IL- β and higher IL-10 production). The relatively higher incorporation of DHA into the lipid rafts of splenocyte membranes coincided with a reduced MHC Class II molecule expression and lower IL-1β production.

4.6 Literature Cited

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5 EFFECTS OF FEEDING FISH OIL ON MESENTERIC LYMPH NODE CYTOKINE RESPONSES IN OBESE LEPTIN RECEPTOR DEFICIENT JCR:LA-*CP* RATS[1]

5.1 INTRODUCTION

It is well established that higher levels of circulating biomarkers of inflammation are present in the obese/insulin resistant state, strongly suggesting an inappropriate immune activation or regulation (as reviewed by (1)). Moreover, individuals with a higher BMI are reported to be at an increased risk of infection and infection-related mortality (as reviewed by (2)), have poor antibody responses to vaccines (3-5) and have immune cells with a reduced capacity to proliferate when stimulated with T-cell mitogens (6, 7). More recently, a higher prevalence of atopic diseases has been reported in the overweight population, suggesting heightened activation of T-helper 2 (Th2) cells (as reviewed by (8)).

Adipose tissue, particularly visceral fat, has been shown to be actively involved in producing and secreting inflammatory mediators, including leptin, adiponectin, monocyte chemoattractant protein-1 (MCP-1), IL-6 and TNF-α (1). However, the impact of visceral adipose tissue accretion on more specific aspects of immune function is not well defined. Evidence to date suggests that immune cells of lymph nodes embedded in adipose tissue can influence adipocyte function and vice versa. Pond *et al* (2002)(9) reported that stimulation of lymph nodes by lipolysaccaride (signals emanating from within the node) *in vivo* can induce lipolysis in surrounding adipocytes. On the other hand, adipocytes are capable of secreting a vast array of adipokines that can influence immune cell function (as reviewed by (10)). Only one study has investigated MLN immune cell function in an obese rodent and Kim *et al* (2008)(11) reported that there was atrophy of MLN in high fat-fed mice and fewer total number of lymphocytes were present in MLN. Collectively, research suggests an effect of obesity/visceral fat on immune cells located in MLN and vice versa; however, the ability of these immune cells to respond to stimulation has not been assessed.

The JCR:LA-*cp* rat is a genetic model of obesity that has a non-functional leptin receptor that prevents any known signal transduction of leptin. Rats that are homozygous for the autosomal recessive *cp* gene (*cp*/*cp*) are obese and insulin resistant, have

[1] A version of this chapter has been submitted for publication to the *International Journal of Obesity*.

dyslipidemia and develop early atherosclerotic lesions (12-14). Consequently, this model has been used extensively to study the impact of metabolic syndrome on various parameters of cardiovascular disease; however, this is the first study to examine aspects of the gut-associated immune system.

It is well established that dietary nutrients, particularly lipids, can influence T cell function and the inflammatory response. Of particular interest in the literature has been the omega-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have potent effects on immunity and inflammation that improve chronic inflammatory conditions (as reviewed by (15)). Furthermore, there is sufficient evidence to suggest that (n-3) PUFA can modify visceral adipose tissue by improving the inflammatory environment and decreasing adiposity (16-18). Other researchers have reported that feeding a diet enriched in EPA and DHA to macrosomic pups of diabetic dams improved the proliferative response of splenocytes to concanavalin A (ConA) and lowered the Th1:Th2 of serum cytokines (19, 20). These studies suggest that feeding fish oil to obese rodents may lower the inflammatory reaction and improve T-cell proliferative responses in the spleen. Hence, the objectives of this study were to determine the influence of obesity on MLN immune cell function and to establish if a diet supplemented with long chain (n-3) PUFA could improve the immune dysfunction present in the gut-associated lymph nodes in the obese JCR:LA-*cp* rat through modifications to the membrane fatty acid composition of MLN immune cells.

5.2 Materials and Methods

5.2.1 Animals and Diet

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee. Male obese (*cp/cp*) and lean (*Cp/Cp* or *Cp/cp*) rats of the JCR:LA-*cp* strain were raised in our breeding colony at the University of Alberta. All animals were individually housed in a temperature and humidity controlled environment with a 12/12-h reversed light cycle.

Animals were weaned at 3 weeks of age and given free access to water and standard laboratory rat chow (Lab diet 5001, PMI Nutrition International, Brentwood, MO). At 12weeks of age 1%w/w cholesterol (Sigma, Oakville, ON, Canada) was added to the rat

chow for 2 wks to accelerate the atherosclerotic process. At 14wks, these rats were randomly allocated to receive one of the following nutritionally complete diets (N=10/diet): control (Ctl, 0% EPA+DHA, 1% w/w cholesterol), low fish oil (LFO, 0.8% w/w EPA+DHA, 1%w/w cholesterol) or high fish oil (HFO, 1.4%w/w EPA+DHA, 1% w/w cholesterol). Lean JCR:LA-*cp* (*Cp*/*cp* or *Cp*/*Cp*) rats (n=5) were fed the Ctl diet. The nutrient composition of the diets is described in Table 5.1 and the fatty acid composition of the dietary fats described in Table 5.2. Diets were prepared weekly and stored at 4°C until fed, feed cups were replaced every 2-3 days to prevent oxidation. Animals were weighed and feed consumption, adjusted for food spillage, was recorded twice weekly. After an overnight fast, rats were killed by cardiac exsanguination under iso-fluorane anaesthesia. Mesenteric lymph nodes (MLN) were removed aseptically.

Table 5.1. Composition of experimental diets.

	Ctl	LFO	HFO
Diet Ingredient		(g/kg)	
Casein (high protein)*	267	267	267
Corn Starch¶	212	212	212
Dextrose *	215	215	215
Non-nutritive cellulose*	79	79	79
Fat Mixture	148	148	148
AOAC Vitamin Mix *	9.4	9.4	9.4
Bernhart-Tomerelli Mineral Mix *	48	48	48
Choline Bitartrate*	2.7	2.7	2.7
Inositol *	6.2	6.2	6.2
L-methionine*	2.5	2.5	2.5
Cholesterol‡	10	10	10
Fat Mixture		g/kg	
Flaxseed Oil§	3	3	3
Stearine††	91	91	94
Sunflower Oil†	54	40	24
Fish Oil **	0	14	27

* Harlan-Teklad (Madison, WI, USA); † Save-On Foods (Edmonton, AB, Canada) ‡ Sigma (Oakville, ON, Canada); § Holistic® Flaxseed oil (London Drugs, Edmonton, AB, Canada); †† Completely hydrogenated soybean oil (CanAmera, Edmonton, AB, Canada); ¶ Safeway (Edmonton, AB, Canada) ** Fish Oil contained 3mg/g mixture of alpha tocopherols (Ocean Nutrition Dartmouth, NS, Canada)

Table 5.2. Fatty acid composition of experimental diets.

	Ctl	LFO	HFO
	g/100 g fatty acids		
14:0	0.08	0.14	0.13
16:0	8.5	11	7.6
18:0	54	54	56
18:1(n-9)	9.3	8.4	5.5
18:2 (n-6)	23	17	17
18:3 (n-6)	0.03	0.00	0.01
18:3 (n-3)	1.3	1.6	1.2
20:5 (n-3)	0.00	3.7	6.4
22:5 (n-3)	0.00	0.02	0.01
24:0	0.15	0.18	0.10
22:6 (n-3)	0.00	1.7	3.0
Total PUFA	25	24	28
Total SFA	65	66	65
PUFA:SFA	0.4	0.4	0.4
Total (n-6) PUFA	24	18	17
Total (n-3) PUFA	1.3	7.0	11
(n-6):(n-3) PUFA	19	3	2
Total EPA+DHA	0.0	5.4	9.4

Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids; EPA, eicosapentaenoic acid (20:5(n-3)); DHA, docosahexaenoic acid (22:6(n-3)).

5.2.2 Isolation of MLN Cells and Primary Culture Conditions

The MLN were placed in sterile 0.5% w/v bovine serum albumin in Krebs-Ringer HEPES buffer (pH 7.4) and immune cells were isolated as we have previously described in detail (21). Isolated MLN immune cells were resuspended in complete culture media [RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic-antibiotic solution, HEPES (25 mmol/L), and 2-mercaptoethanol (2.5 mmol/L), Invitrogen, Burlington, ON, Canada] and counted on a hemacytometer (Fisher Scientific, Edmonton, AB, Canada). MLN cells ($1.25x10^6$ cells/L) were resuspended in the culture media described above and incubated in 4 mL sterile polystyrene tubes (Fisher Scientific Company, Ottawa, ON, Canada) in a humidified atmosphere at 37°C in the presence of 5% v/v CO_2. The cell culture medium contained either no mitogen (unstimulated cells) or ConA (2.5mg/L, ICN, Montreal, PQ, Canada) or pokeweed

mitogen (PWM) (55mg/L, Sigma-Aldrich, Oakville, ON, Canada). After 48 h of culture, the supernatant was removed and stored at -80°C until cytokine assays were performed. Cell pellets were washed with PBS, re-pelleted and liquid was removed prior to storing at -80°C for fatty acid analysis.

5.2.3 Phenotype Analysis

MLN immune cell subsets were identified by one, two or three colour direct immunofluorescence assay as we have previously described.(22) The following pre-labelled mAbs were used: CD3 and CD28 (FITC-labelled); CD4, CD8, CD3, CD11b/c and OX12 (PE-labelled); and CD25 and CD8 (biotin-labelled). All antibodies were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada) except CD4, and OX12, which were purchased from Serotec (Cedarlane Laboratories Ltd, Hornby, ON, Canada). Streptavidin-Quantum Red™ (R-PE-Cy5 fluorochrome, Sigma-Aldrich, Oakville, ON, Canada) was added to wells containing biotin-labelled Ab. Cell fixative (200 µl of 1% w/v paraformaldehyde) was added to each well after final wash. The proportion of cells that were positive for each marker was determined by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA) according to the relative fluorescence intensity using CellQuest software (Becton Dickinson, Sunnyvale, CA).

5.2.4 Cytokine Production and Serum Haptoglobin

All of the following assays were performed according to the manufacturer's instructions. The cultured cell supernatants of unstimulated, ConA and PWM-stimulated MLN cells were used to determine IL-1β and TNF-α (detection limit: 31.2-2000pg/mL), IL-2 (23.4-1500 pg/mL), IL-4 (1.6-100 pg/mL), IL-6 (78-5000 pg/mL), IL-10 (15.6-1000 pg/mL) and IFN-γ (31.25-2000 pg/mL) levels with OptEIA anti-rat enzyme-linked immunoasorbant assay (ELISA) (BD Biosciences, PharMingen, Mississauga, ON, Canada). Values below the range of detection were assigned half of the value of the lowest standard value. Serum haptoglobin levels were determined by a colorimetric assay purchased from Tri-Delta Development Limited (Maynooth, Ireland) according to the manufacturer's instructions. All samples were measured in duplicate and the absorbance for cytokine assays was measured at 450nm and 630nm for haptoglobin on a microtitre plate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA). The average of

the duplicate data was used for statistical analysis if the coefficient of variance was ≤ 10%.

5.2.5 MLN Phospholipid Fatty Acid Composition

A modified Folch method was used to extract lipids from isolated MLN cells(23) and total phospholipids were isolated from whole cells on silica G plates(24). Fatty acid methyl esters were prepared from total phospholipids(23) and were separated by automated gas liquid chromatography (Varian 3800, Varian Inc., Mississauga, ON, Canada) using a 100m *CP*-Sil 88 fused capillary column (Varian Inc., Mississauga, ON, Canada) as described elsewhere (25).

5.2.6 Statistics

Statistical analysis was conducted using the SAS software statistical package (Version 9.1, SAS Institute, Cary, NC). All data were reported as mean ± SEM. Significant differences among groups were determined by Duncan's multiple range test ($p<0.05$) and all non-parametric data were log-transformed prior to statistical analyses. Differences between groups with data that remained nonparametric after log-transforming were analyzed using Kruskal-Wallis/Wilcoxon ($p<0.05$).

5.3 Results

5.3.1 Feed Intake and Body Weight

Obese rats fed the Ctl diet had higher daily feed intake (33 ± 0.4 g/d vs. 20 ± 0.3 g/d, $p<0.05$) and final bodyweights (592 ± 5 g vs. 378 ± 4 g, $p<0.05$) compared to lean rats. Obese rats fed LFO (556 ± 16 g) or HFO (561 ± 6 g) diet had lower final body weights than the obese rats fed the Ctl diet (592 ± 5 g, $p<0.05$), but average daily feed intake did not differ (34 ± 0.6 g/d, LFO or 32 ± 0.3 g/d, HFO).

5.3.2 Phospholipid Fatty Acid Composition of MLN Cells

The relative proportions of fatty acids from 14:0 to 24:1 (n-9) in MLN phospholipids are reported in Table 5.3. Compare to lean rats, obese rats fed the Ctl diet had a higher proportion of 16:0, 16:1*trans*9, 16:1 (n-9), 18:1(n-7), 20:3(n-6), 20:4(n-3), 22:1(n-9), 20:5(n-3), 22:3(n-3), 22:5(n-3), 22:6(n-3) and total MUFA and total (n-3) PUFA and a lower proportion of C18:0, 20:2(n-6), 20:4(n-6), 24:1(n-9) and total (n-6) PUFA and (n-6):(n-3) PUFA ratio ($P<0.05$).

Compared to Ctl-fed obese rats, obese rats fed either fish oil diet had a higher proportion of 18:0, 18:1(n-9), 18:2(n-6), 20:5(n-3), 22:5(n-3), and total (n-3) PUFA and had a lower proportion of 20:2(n-6), 20:4(n-6), 24:1(n-9), total PUFA, PUFA:SFA ratio, (n-6) PUFA and (n-6):(n-3) ratio ($P<0.05$). Only obese rats fed the HFO diet had significantly higher proportions of 16:0, 16:1(n-9), 18:1(n-7), 22:6(n-3) and lower proportions of 20:2(n-6), 20:4(n-3) and 22:3(n-3) in comparison to obese rats fed the Ctl diet ($P<0.05$). Compared to the LFO group, HFO rats had a greater proportion of 16:1(n-9), 18:1(n-7), 20:5(n-3), 22:5(n-3), total n-3 PUFA and a smaller proportion of 20:4(n-3), 20:4(n-6), 24:1(n-9) and total (n-6) PUFA and (n-6):(n-3) PUFA ratio ($P<0.05$, Table 5.3).

Table 5.3. Fatty acid composition of MLN immune cell phospholipids in lean rats fed the Ctl diet and obese rats fed the Ctl, LFO or HFO diet.

	Lean Ctl	Obese Ctl	LFO	HFO
		g/100g		
C14:0	0.43 ± 0.08	0.47 ± 0.06	0.54 ± 0.08	0.58 ± 0.06
C16:0	19 ± 1^{b}	22 ± 0^{c}	22 ± 1^{ab}	24 ± 0^{a}
C16:1(n-9)	0.34 ± 0.07^{c}	0.8 ± 0.01^{b}	0.87 ± 0.03^{b}	1.1 ± 0.04^{a}
C18:0	25 ± 1^{a}	21 ± 0^{c}	23 ± 1^{b}	23 ± 1^{b}
C18:1(n-9)	7.2 ± 0.1^{c}	7.5 ± 0.1^{c}	8.5 ± 0.1^{b}	9.3 ± 0.3^{a}
C18:1(n-7)	2.0 ± 0.1^{c}	3.0 ± 0.1^{b}	3.2 ± 0.1^{ab}	3.4 ± 0.1^{a}
C18:2(n-6)	10 ± 0^{b}	10 ± 0^{b}	12 ± 0^{a}	12 ± 0^{a}
C18:3(n-3)	0.97 ± 0.04	0.85 ± 0.03	0.94 ± 0.04	0.85 ± 0.04
C20:2(n-6)	1.8 ± 0.1^{a}	1.2 ± 0.0^{b}	1.1 ± 0.1^{b}	0.7 ± 0.0^{c}
C20:3(n-3)	1.5 ± 0.1^{b}	1.9 ± 0.1^{a}	1.9 ± 0.1^{a}	1.5 ± 0.1^{b}
C20:4(n-6)	26 ± 0^{a}	23 ± 1^{b}	16 ± 0^{c}	13 ± 0^{d}
C20:5(n-3)	ND	0.3 ± 0.01^{c}	1.9 ± 0.0^{b}	4.0 ± 0.3^{a}
C24:0	ND	0.42 ± 0.04	0.35 ± 0.06	0.44 ± 0.01
C24:1(n-9)	2.7 ± 0.1^{a}	1.8 ± 0.0^{b}	0.84 ± 0.04^{c}	0.47 ± 0.02^{d}
C22:5(n-3)	0.48 ± 0.04^{d}	1.1 ± 0.1^{c}	2.2 ± 0.1^{b}	2.8 ± 0.1^{a}
C22:6(n-3)	0.83 ± 0.05^{c}	1.6 ± 0.1^{b}	1.6 ± 0.1^{ab}	1.9 ± 0.1^{a}
MUFA	13 ± 0^{b}	14 ± 0^{a}	15 ± 0^{a}	15 ± 0^{a}
SFA	45 ± 0^{bc}	43 ± 1^{c}	46 ± 1^{ab}	47 ± 0^{a}
PUFA	42 ± 0^{a}	41 ± 1^{a}	39 ± 1^{b}	37 ± 1^{b}
PUFA:SFA	0.93 ± 0.01^{a}	0.97 ± 0.03^{a}	0.84 ± 0.03^{b}	0.78 ± 0.02^{b}
(n-6) PUFA	38 ± 0^{a}	36 ± 1^{a}	32 ± 1^{b}	28 ± 1^{c}
(n-3) PUFA	3.8 ± 0.1^{d}	6.4 ± 0.2^{c}	9.0 ± 0.2^{b}	11 ± 0^{a}
(n-6):(n-3)	10 ± 0^{a}	5.6 ± 0.1^{b}	3.5 ± 0.1^{c}	2.5 ± 0.1^{d}

Data represent mean ± SEM; n=9/group for LFO, n=8/group for Ob Ctl and HFO, and n=4/group for lean rats. Means within the same row that do not share a common letter are significantly different ($p<0.05$). The relative proportions of fatty acids from 12:0 to 24:1(n-9) were measured in MLN phospholipid membrane, but only the major fatty acids were reported. Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

5.3.3 Phenotypes of MLN Immune Cells

Obese rats fed the control diet had a higher proportion of MLN cells that were $CD3^+CD8^+$ (cytotoxic T-cells), and $CD11b/c^+OX6^-$ (monocytes) and a lower proportion of $CD4^+CD25^+$ (T-helper cells expressing the IL-2 receptor) compared to lean rats fed the control diet (Table 5.4, $P<0.05$). The proportion of $CD4^+$ cells that expressed CD25 did not differ between lean (16 ± 0) and obese rats (15 ± 1) fed the control diet. Obese rat of the FO-supplemented groups, had a lower proportion of $CD8^+CD25^+$ (cytotoxic T-cells expressing the IL-2 receptor) MLN cells than obese rats fed the Ctl diet. However, although only those rats fed the LFO diet had a lower percentage of $CD8^+$ cells that expressed $CD25^+$ (34 ± 1 compared to 47 ± 3 for Ob Ctl, not presented in Table 5.4). Rats fed the LFO diet had a lower proportion of $CD11b/c^+$ and $CD11b/c^+OX6^+$ (non-B-cell antigen presenting cells expressing the major histocompatibility complex [MHC] II) MLN cells. FO did not alter the proportion of $OX12^+$ (B-cells), $OX6^+$ (antigen presenting cells), $OX6^-CD11b/c^+$, $OX6^+CD11b/c^-$, $CD4^+CD28^+$ (T-helper cells expressing co-stimulatory molecule), $CD8^+CD28^+$ (cytotoxic T-cells expressing co-stimulatory molecule), $CD4^+CD25^+$, $CD3^+$ (T-cells), $CD3^+CD8^+$ or $CD3^+CD4^+$ cells.

Table 5.4. MLN immune cell phenotypes of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA-*cp* rats fed the Ctl, LFO or HFO diet.

	Lean Ctl	Obese Ctl	LFO	HFO
	% of total gated cells			
$CD3^+$	70 ± 1	68 ± 1	67 ± 1	68 ± 1
$CD4^+$	69 ± 1	68 ± 1	67 ± 1	68 ± 1
$CD3^+CD4^+$	54 ± 2^b	56 ± 1^{ab}	57 ± 1^{ab}	58 ± 1^a
$CD8^+$	11 ± 0^b	18 ± 1^a	16 ± 0^a	17 ± 1^a
$CD3^+CD8^+$	11 ± 0^b	16 ± 1^a	15 ± 0^a	15 ± 1^a
$CD4^+CD25^+$	9.0 ± 0.4^a	6.0 ± 0.3^b	6.1 ± 0.4^b	7.2 ± 0.5^b
$CD8^+CD25^+$	2.5 ± 0.1^{ab}	2.9 ± 0.3^a	1.9 ± 0.1^b	2.0 ± 0.2^b
$CD4^+CD28^+$	ND	64 ± 2	65 ± 1	67 ± 2
$CD8^+CD28^+$	ND	9.0 ± 0.5	7.7 ± 0.6	9.6 ± 0.8
$OX6^+$	29 ± 1	31 ± 2	28 ± 2	29 ± 1
$CD11B/C^+$	4.9 ± 0.2^{ab}	5.7 ± 0.7^a	3.8 ± 0.2^b	5.0 ± 0.5^{ab}
$OX6^+CD11b/c^+$	4.5 ± 0.2^a	4.7 ± 0.5^a	3.1 ± 0.2^b	4.1 ± 0.3^a
$OX6^+CD11b/c^-$	25 ± 1^b	26 ± 2^a	25 ± 2^{ab}	25 ± 1^a
$OX12^+$	24 ± 1	25 ± 1	25 ± 2	23 ± 1

Data represent mean ± SEM; n=10/group for obese rats and n=5/group for lean rats. Values are a proportion of the total gated cells as determined by immunofluorescence. Means within the same row that do not share a common letter are significantly different ($p<0.05$). ND, not determined.

5.3.4 Cytokine production of MLN Immune Cells

Obese rats fed the control diet produced more IL-1β, and IL-10 when MLN immune cells were stimulated with PWM and more IL-4 when stimulated with ConA compared to lean rats fed the same diet (Table 5.5). Obese rats fed either the LFO or HFO diet had lower ConA-stimulated IL-4 production compared to obese rats fed the Ctl diet. However, only obese rats fed the LFO diet had lower ConA-stimulated IL-10 production and only obese rats fed the HFO diet had lower PWM-stimulated IL-1β. Production of IL-10 in unstimulated MLN immune cells did not differ among groups (Table 5.5). There were no detectable levels of TNF-α, IFN-γ, IL-4 or IL-6 in the supernatant of unstimulated MLN immune cells.

Table 5.5. MLN immune cell mitogen-stimulated cytokine production of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA-*cp* rats fed the Ctl, LFO or HFO diet.

		Ctl Lean	Ctl Obese	LFO	HFO
			(pg/ml)		
ConA	IL-2	335 ± 102	500 ± 143	423 ± 51	294 ± 49
	IL-4	3.7 ± 0.3^{b}	6.7 ± 0.9^{a}	3.8 ± 0.8^{b}	3.9 ± 0.8^{b}
	IFN-γ	118 ± 30	185 ± 27	168 ± 46	175 ± 50
	IL-10	266 ± 26^{ab}	295 ± 24^{a}	175 ± 27^{b}	214 ± 54^{ab}
PWM	IFN-γ	152 ± 64^{ab}	234 ± 47^{a}	136 ± 37^{ab}	82 ± 21^{b}
	IL-1β	38 ± 2.4^{b}	78 ± 15^{a}	55 ± 9^{ab}	43 ± 5^{b}
	IL-10	291 ± 15^{b}	452 ± 31^{a}	368 ± 30^{ab}	350 ± 39^{ab}
UNS	IL-10	38 ± 3	56 ± 7	51 ± 6	53 ± 5

Data represent mean ± SEM; n=10/group for obese rats and n=5/group for lean rats. Means within the same row that do not share a common letter are significantly different ($p<0.05$).

5.3.5 Haptoglobin

Serum concentrations of haptoglobin were higher in obese Ctl rats (1.7 ± 0.1 mg/ml) compared to lean Ctl rats (0.84 ± 0.11 mg/ml) ($P<0.05$). The concentration of serum haptoglobin in either the LFO-fed rats (1.6 ± 0.1 mg/ml) or HFO-fed rats (1.6 ± 0.1 mg/ml) did not differ from obese Ctl rats ($P<0.05$).

5.4 Discussion

This is the first study to investigate the effect of diet and obesity on the MLN cells, part of GALT, in the JCR:LA-*cp* rat, an established model of the metabolic syndrome. Our findings demonstrate that MLN of obese rats have a heightened Th2 cytokine (IL-4 and IL-10) response and produce higher levels of an inflammatory cytokine (IL-1β) after mitogen stimulation. Furthermore, feeding diets containing EPA and DHA normalized these responses to levels similar to lean rats and decreased weight gain in the JCR:LA-*cp* rat, without affecting the proliferative response (IL-2 production) of lymphocytes to a T-cell mitogen.

5.4.1 Fatty Acid Composition of MLN Phospholipids

The fatty acid composition of MLN immune cell phospholipid membranes in obese JCR:LA-*cp* rats differed significantly from lean rats fed the same diet. Obese rats fed the control diet had a lower (n-6):(n-3) PUFA ratio due to a greater incorporation of total (n-3) fatty acids, including EPA, docosapentaenoic acid and DHA, and a lower incorporation of total (n-6) PUFA, including AA. The lower (n-6):(n-3) PUFA ratio in MLN phospholipids is consistent with our findings in splenocyte phospholipids of *fa/fa* Zucker rats (26). The literature is supportive that increasing dietary (n-3) PUFA lowers the (n-6):(n-3) PUFA ratio in the phospholipid membrane of inflammatory immune cells. These observations also suggest that a lower amount of arachidonic acid is available for inflammatory eicosanoid production (as reviewed by (15)). Consistent with this, several studies conducted in chronic states of inflammation have reported that feeding fish oil lowers the production of inflammatory cytokines, including IL-1β (27, 28). Furthermore, fish oil has potentially beneficial effects in conditions with heightened Th2 cytokine responses (as reviewed by (29)). However, despite a lower (n-6):(n-3) PUFA ratio in MLN immune cell phospholipids in obese JCR:LA-*cp* rats, mitogen-stimulated production of an inflammatory cytokine and Th2 cytokines was still higher, suggesting that additional factors contribute to the cytokine response in this animal model. It is possible that the chronic inflammatory state, as evidenced by higher circulating haptoglobin in obese JCR:LA-*cp* rats, and/or the adipose tissue environment may partly explain the altered cytokine response of MLN immune cells in obese rats. Furthermore, this data suggests that the JCR:LA-*cp* rat has altered essential fatty acid metabolism .

5.4.2 Potential Influence of Adipose Tissue on MLN Immune Cells

Recent evidence indicates that adipose tissue, particularly visceral fat, is actively involved in producing and secreting inflammatory mediators (1). To date, only one study has investigated the impact of obesity and factors secreted by adipose tissue on MLN immune cell function. Kim *et al* (2008)(11) reported that high fat fed mice had smaller MLN and subsequently fewer immune cells. The authors suggested that factors emanating from the mesenteric fat, such as free fatty acids, induced apoptosis of lymphocytes residing in MLN (11). In this study, we also demonstrate that immunity in gut-associated lymph tissue is altered. More specifically, MLN immune cells of obese JCR:LA-*cp* rats produce higher amounts of IL-1β, suggesting a heightened inflammatory response as compared to peripheral immune cells and are likely influenced by the inflammatory environment of the mesenteric adipose tissue. Of the cell types we examined (by flow cytometry) there were no differences in the proportion of cell types in the MLN between lean and obese Ctl-fed rats. This indicates that that the higher production of IL-1β in obese JCR:LA-*cp* rats is likely due to a functional change in MLN immune cells.

5.4.3 Cytokine Production

MLN immune cells of obese JCR:LA-*cp* rats produced significantly more of the Th2 cytokines, IL-4 and IL-10 compared to lean rats fed the same control diet. Other studies have reported that mitogen-stimulated IL-4 and OVA-stimulated IL-2 production of splenocytes was higher in obese high fat fed mice.(30, 31) Contrary to the JCR:LA-*cp* rat, lower T-cell mitogen-stimulated proliferation and IL-2 production in splenocytes of has been reported in *fa/fa* Zucker rats(26, 32, 33), diet-induced obese (DIO) mice(30) and DIO rats(34, 35). Obese JCR:LA-*cp* rats also produced more PWM-stimulated IL-10, but production did not differ with ConA stimulation compared to lean Ctl rats. The production of IL-10 and IL-4 was higher in Ctl-fed obese rats, despite a lower total proportion of T-helper cells expressing the IL-2 receptor ($CD4^+CD25^+$), a major source of these cytokines. The lower proportion of $CD4^+CD25^+$ MLN cells in obese JCR:LA-*cp* rats is supported by a study conducted in high fat fed obese mice.(11) This suggests that factors other than the proportion of T-helper cells present in the MLN contributed to the heightened Th2 cytokine response. The *ex vivo* stimulated cytokine production in this

study suggests that MLN (major site of antigen exposure) immune cells of obese JCR:LA-*cp* rats would respond to a T cell challenge with a greater Th2 type response. This heightened Th2 cytokine response may have negative implications on development of atopic diseases shown to be elevated in the overweight population (as reviewed by (8)).

5.4.4 Potential contribution of a Leptin Receptor Defect

Despite the lower (n-6):(n-3) PUFA ratio and absence of leptin signal via the long form of the leptin receptor (Ob-Rb), obese JCR:LA-*cp* rats produced more mitogen-stimulated IL-1β and production of IL-2 did not differ from lean rats. These findings were unexpected in light of evidence that suggests leptin is critical for the proliferative response of $CD4^+$ T-lymphocytes, prevention of T-lymphocyte apoptosis and secretion of IL-2 as well as regulation of the inflammatory response of both the innate and acquired immune systems (as reviewed by (36)). Our data implies that leptin signalling via the leptin receptor is not critical for adequate IL-2 or IL-1β secretion in mitogen-stimulated MLN immune cells in the obese JCR:LA-*cp* rat.

5.4.5 Effects of Feeding Fish Oil on MLN Immune Cell Function

Feeding fish oil to obese JCR:LA-*cp* rats could be interpreted as having a favourable effect on IL-4 and IL-10 production and those rats fed the higher level of EPA and DHA produced less of an inflammatory cytokine (IL-1β). Although knowledge of the effects of dietary (n-3) PUFA on immune function in obesity are limited, Khan *et al* (2006)(20) reported contrary to our findings that overweight offspring fed a high (n-3) PUFA diet had lower mRNA levels of IFN-γ in spleen and higher mRNA levels of IL-4. The apparent disparity between our study and Khan's may be due to the initial higher IFN-γ and lower IL-4 mRNA spleen levels, the much lower dietary (n-3):(n-6) PUFA, the tissue examined and the younger age of the rodents studied in Kahn *et al*'s study.(20) Similar to our study in which IL-4 production was elevated, it has been reported that feeding (n-3) PUFA can lower IL-4 levels in a rodent model of contact dermatitis (37). Although the literature is inconsistent, there is some evidence that suggests dietary fish oil can lower serum IL-10 levels in a model of experimental rheumatoid arthritis, a state with high serum IL-10 (38) and can lower IL-10 in response to allergen in susceptible neonates (39). In the current study, feeding FO did not significantly modify IL-2

production in MLN cells stimulated with a polyclonal T cell mitogen (ConA), indicating that increasing dietary (n-3) PUFA does not suppress T-cell function in obese JCR:LA-*cp* rats which has been reported in healthy animals (40-43). Overall, our study is supportive of the concept that fish oil may be beneficial in skewed Th2 states, as observed in the gut-associated lymph tissue of obese JCR:LA-*cp* rats.

The lower IL-1β production observed in immune cells of the MLN isolated from obese rats fed the HFO diet is consistent with the lower (n-6):(n-3) PUFA ratio in MLN phospholipids. As stated previously, increasing the content of (n-3) PUFA in inflammatory cells can reduce the production of inflammatory mediators (as reviewed by (15)). Other effects of feeding (n-3) PUFA may have contributed to the lower inflammatory response of MLN immune cells, including decreased weight gain. Additionally, recent studies have demonstrated that feeding (n-3) PUFA can lower inflammatory mediator production of visceral adipose tissue(16, 18), which may have had an impact on the inflammatory response observed in the current study. Of the cell types examined (by flow cytometry), the HFO diet did not affect immune cell phenotypes that would explain the lower IL-1β production, suggesting that the higher level of (n-3) PUFA modifies functional aspects of MLN immune cells.

5.5 Conclusion

Our results demonstrate that mitogen-stimulated cytokine production from MLN immune cells is altered in the obese JCR:LA-*cp* rat, an established model of metabolic syndrome. Immune cells isolated from MLN of obese rats produced significantly more Th2 cytokines, IL-4 and IL-10 and an inflammatory cytokine, IL-1β, despite a higher proportion of (n-6):(n-3) PUFA in MLN phospholipids. In comparison to lean rats, obese rats produced similar levels of IL-2 in response to a T-cell mitogen, even with a lower proportion of $CD4^+CD25^+$ T-cells. Feeding either the low or high fish oil diets had a favourable effect on body weight gain. Moreover, feeding fish oil to obese JCR:LA-*cp* rats normalized IL-4 and IL-10 production from MLN immune cells, without affecting ConA stimulated IL-2 production. However, only the HFO diet significantly lowered IL-1β production, which indicates a higher level of EPA and DHA may be required to improve the inflammatory response in obese rats. This is the first study to report that feeding fish oil to obese, leptin receptor deficient JCR:LA-*cp* rats normalizes impaired

cytokine response of immune cells of mesenteric lymph nodes residing in visceral adipose tissue. Moreover, these modifications are likely mediated independent of leptin signalling.

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6 EFFECTS OF OBESITY AND DIETARY LONG CHAIN (N-3) POLYUNSATURATED FATTY ACIDS ON IL-2 PRODUCTION AND PKC-θ LEVELS IN OBESE INSULIN RESISTANT JCR:LA-*CP* RATS[1]

6.1 INTRODUCTION

Obesity is one of the leading health crises facing the global community, particularly due to the associated risk of a higher body mass index (BMI) with other chronic diseases. Altered or impaired immune responses have been identified in overweight individuals and these have been implicated in the pathogenesis of several co-morbidities of obesity. Obesity is generally considered a chronic inflammatory state and there is a greater incidence of other inflammatory-associated including cardiovascular disease, type 2 diabetes and certain forms of cancer (1, 2) in overweight adults. In addition, alterations in the acquired immune system have also been reported in obese individuals, including impaired antibody responses to vaccination (3-5) and reduced proliferative responses of B and T-lymphocytes (6-8).

The JCR:LA-*cp* rat is a genetic model of obesity that expresses a dysfunctional leptin receptor which prevents any known receptor (Ob-R) mediated signal of leptin. Rats that are homozygous for the autosomal recessive *cp* gene (*cp*/*cp*) are obese and insulin resistant, have dyslipidemia and develop atherosclerosis (9-11). This animal model has been used extensively to study the impact of metabolic syndrome on various parameters of cardiovascular disease (12, 13). Recently we reported alterations in immune health in this rodent model (14). T-cells have been implicated in the pathogenesis of inflammation and atherosclerosis and we and others have reported that the ability of T-cells to produce IL-2 and/or proliferate in response to T-cell mitogens is impaired in obese states (15-24). Thus, we sought to determine the impact of obesity on T-cell function in JCR:LA-*cp* rats.

It is well established that feeding the (n-3) PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), influence immunity and inflammation and improve health outcomes in chronic inflammatory diseases (as reviewed by (25)). Investigations into the impact of dietary (n-3) PUFA on immune health in overweight adults are limited. Collectively, these feeding studies have reported that supplementing mixtures of EPA and DHA (1.1-4.2g/d) for a short duration (6-12wk) have only a limited effect on systemic

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markers of inflammation in obese men or women (26-30), and offer little insight into the direct impact of long chain (n-3) PUFA on immune cell function. Fish oil (FO)-supplementation in rodent models of obesity are also limited, but suggest that FO may improve T-cell function in obesity (19, 31). One research group, using macrosomic offspring of diabetic rat dams, reported that feeding EPA and DHA improved the proliferative response of splenocytes to ConA (19) and lowered the Th1:Th2 of serum cytokines and spleen mRNA expression (31). Guermouche *et al* (2004) (19) reported that improvements in intracellular calcium homeostasis was responsible for the restored proliferative response of splenocytes. The underlying mechanisms that are modified by (n-3) PUFA in animal models of obesity have not been established.

Protein kinase C-theta (PKC-θ) is a serine/threonine kinase expressed only in T-lymphocytes, platelets and muscle tissue (as reviewed by (32)). Successful activation of T-lymphocytes via the T-cell receptor (TCR) results in recruitment of PKC-θ to the immunological synapse, which subsequently activates transcription factors integral to IL-2 synthesis. Based on *in vitro* studies in PKC-θ deficient mice, this activation is essential for IL-2 production by T-cells (33). It has been demonstrated that feeding DHA can modify the amount of PKC-θ in lipid rafts, which subsequently prevented optimal IL-2 secretion in $CD4^+$ T-lymphocytes (34). The purpose of this study was to determine the effect of obesity and dietary FO on PKC-θ levels in splenocytes activated with a polyclonal T-cell mitogen.

6.2 Materials and Methods

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Agricultural, Life and Environmental Sciences Animal Ethics Committee at the University of Alberta. Male obese (*cp/cp*) and lean (*Cp/Cp* or *Cp/cp*) rats of the JCR:LA-*cp* strain were raised in our breeding colony at the University of Alberta. All animals were individually housed in a temperature and humidity controlled environment with a 12/12-h reversed light cycle.

Animals were weaned at 3 weeks of age and given free access to water and standard laboratory rat non-purified diet (Lab diet 5001, PMI Nutrition International, Brentwood, MO, USA). At 8 wks, *cp/cp* rats were randomly allocated to receive one of the following nutritionally complete diets (n=8/diet) for 16 wks: control (Ctl, 0%

EPA+DHA, 1% w/w cholesterol), low FO (LFO, 0.8% w/w EPA+DHA, 1%w/w cholesterol) or high FO (HFO, 1.4%w/w EPA+DHA, 1% w/w cholesterol); lean (*Cp/Cp* or *Cp/cp*) rats (n=8) were allocated to the Ctl diet for 16wks. The nutrient composition of the experimental diets is provided in Table 6.1 and the fatty acid composition of the fat included in these diets is provided in Table 6.2. Fresh batches of diet containing oil were prepared weekly and stored at 4°C until fed. Feed cups were replaced every 2-3 days to ensure that the lipid did not oxidize. Rats were weighed and feed consumption, adjusted for food spillage, was recorded twice weekly. After an overnight fast, rats were killed by cardiac exsanguination under iso-fluorane anaesthesia. Blood was collected via cardiac puncture in BD Vacuntainer® (BD Biosciences, Mississauga, ON, Canada) and serum stored at -80°C until later analysis. The spleen was removed under aseptic conditions.

Table 6.1. Composition of experimental diets.

	Ctl	LFO	HFO
Diet Ingredient		g/kg	
Casein (high protein)*	267	267	267
Corn Starch¶	212	212	212
Dextrose *	215	215	215
Non-nutritive cellulose*	79	79	79
Fat Mixture	148	148	148
AOAC Vitamin Mix *	9.4	9.4	9.4
Bernhart-Tomerelli Mineral Mix *	48	48	48
Choline Bitartrate*	2.7	2.7	2.7
Inositol *	6.2	6.2	6.2
L-methionine*	2.5	2.5	2.5
Cholesterol‡	10	10	10
Fat Mixture		g/kg	
Flaxseed Oil§	3	3	3
Stearine††	91	91	94
Sunflower Oil†	54	40	24
Fish Oil **	0	14	27

* Harlan-Teklad (Madison, WI). † Save-On Foods (Edmonton, AB, Canada); ‡ Sigma (Oakville, Canada);§ Holistic Flaxseed oil (London Drugs, Edmonton AB, Canada); ¶ Completely hydrogenated soybean oil (CanAmera, Edmonton, AB, Canada); ** Safeway (Edmonton, AB, Canada); ††Fish Oil contained 3mg/g mixture of alpha tocopherols (Ocean Nutrition Dartmouth, NS, Canada).

Table 6.2. Fatty acid composition of experimental diets.

	Ctl	LFO	HFO
	g/100 g fatty acids		
14:0	0.08	0.14	0.13
16:0	8.5	11	7.6
18:0	54	54	56
18:1(n-9)	9.3	8.4	5.5
18:2 (n-6)	23	17	17
18:3 (n-6)	0.03	0.00	0.01
18:3 (n-3)	1.3	1.6	1.2
20:5 (n-3)	0.00	3.7	6.4
22:5 (n-3)	0.00	0.02	0.01
24:0	0.15	0.18	0.10
22:6 (n-3)	0.00	1.7	3.0
Total PUFA	25	24	28
Total SFA	65	66	65
PUFA:SFA	0.4	0.4	0.4
Total (n-6) PUFA	24	18	17
Total (n-3) PUFA	1.3	7.0	11
(n-6):(n-3) PUFA	19	3	2
Total EPA+DHA	0.0	5.4	9.4

Abbreviations used: SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

6.2.1 Isolation of Splenocytes and Primary Culture Conditions

The spleens were weighed and placed in sterile 0.5% w/v bovine serum albumin in Krebs-Ringer HEPES buffer (pH 7.4) and splenocytes were isolated as we have previously described in detail (35). Isolated splenocytes were resuspended in complete culture media (RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic-antibiotic solution, HEPES (25 mmol/L), and 2-mercaptoethanol (2.5 mmol/L)) and counted on a hemacytometer (Fisher Scientific, Edmonton, AB, Canada). Splenocytes ($1.25x10^6$ cells/L) were resuspended in the culture media described above and incubated in 4 mL sterile polystyrene tubes in a humidified atmosphere at 37°C in the presence of 5% v/v CO_2. Splenocytes were cultured without mitogen (unstimulated cells) or with ConA (2.5mg/L) or LPS (1mg/L). After 48 h of

culture, the supernatant was removed and stored at -80° C until cytokine assays were performed. The cell pellets were washed with PBS, re-pelleted and liquid was removed prior to storing at -80°C for future fatty acid analysis.

Splenocytes ($1.25x10^6$ cells/L) were resuspended and incubated in the culture media described above with or without ConA (2.5mg/L) for 18 h in 4 mL sterile polystyrene tubes in a humidified atmosphere at 37°C in the presence of 5% v/v CO_2. After 18 h, the supernatant was removed and the cell pellets were washed with PBS and re-pelleted. Cells were used either immediately for phenotype analysis or frozen for protein analysis as described in the following sections.

6.2.2 Protein Extraction

Cytoplasmic protein lysates were prepared by resuspending the cell pellets in 400µl of lysis buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.625% NP-40, ddH_2O) with freshly added protease and phosphatase inhibitor cocktails (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), vortexed and incubated on ice for 15 min. Samples were centrifuged for 1 min at 15,000 rpm at 4°C and the supernatant (cytoplasmic extract) was placed in chilled microcentrifuge tubes and stored at -80°C until needed.

6.2.3 Western Blot Analysis

Protein concentrations of cytoplasmic lysates were determined by bicinchoninic acid (BCA) assay (Sigma-Aldrich, Oakville, ON, Canada). Western blotting was performed as previously described with the following modifications (36). The primary monoclonal antibodies, PKC-θ, (AbCam, Cambridge, MA) and Phospho-PKC-θ (Cell Signaling, New England Biolabs, Pickering, ON, Canada) were diluted 1:1000 in TBST (10 mMTris–HCl pH 7.4, 150 mMNaCl, 0.1%v/v Tween-20) and 5% w/v bovine serum albumin (BSA, Sigma-Aldrich, Oakville, ON, Canada) and β-actin (internal control) was diluted 1:25000 in TBST and 5% w/v powdered non-fat milk. Diluted primary antibodies were incubated with the respective nitrocellulose membranes at 4°C overnight. Horseradish peroxidase–conjugated secondary antibodies anti-rabbit IgG was purchased from Cell Signaling Technology (New England Biolabs, Pickering, ON, Canada) and anti-mouse IgG1 was purchased from Caltag Laboratories (Invitrogen, Burlington, ON, Canada). Bands were developed using enhanced chemiluminescence (ECL) detection kit (ECL Plus, Amersham, GE Healthcare, Piscataway, NJ) and visualized bands were captured by the

Typhoon Imaging System (GE Healthcare, Piscataway, NJ). The relative intensities of the bands were determined by ImageQuant software and corrected for beta-actin.

6.2.4 Phenotype Analysis

Immune cell subsets in freshly isolated splenocytes and 18 h post ConA stimulated splenocytes were identified by one, two or three colour direct immunofluorescence assay as we have previously described (37). The following pre-labelled mAbs were used: CD3, OX6 and CD28 (FITC-labelled); CD4 (APC-labelled); CD8, CD11b/c and OX12 (PE-labelled); and CD25 and CD80 (biotin-labelled). All antibodies were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada) except CD4, and OX12, which were purchased from Serotec (Cedarlane Laboratories Ltd, Hornby, ON, Canada) and Streptavidin-Quantum Red™ was purchased from Sigma-Aldrich (Oakville, ON, Canada). Streptavidin-Quantum Red™ (R-PE-Cy5 flurochrome) was added to wells containing biotin-labelled Ab. After final wash, plates were aspirated and 200 µL of cell fix (1% w/v paraformaldehyde) was added to each well. The proportion of cells that were positive for each marker was determined by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA) according to the relative fluorescence intensity using CellQuest software (Becton-Dickinson).

6.2.5 Cytokine Production

All of the following assays were performed according to the manufacturer's instructions. The cultured cell supernatants of unstimulated, ConA and LPS splenocytes were used to determine IL-1β and TNF-α (detection limit: 31.2-2000pg/mL), IL-2 (23.4-1500 pg/mL), IL-6 (78-5000 pg/mL), IL-10 (15.6-1000 pg/mL) and IFN-γ (31.25-2000 pg/mL) levels with OptEIA anti-rat enzyme-linked immunoasorbant assay (ELISA) sets were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada). Values below the range of detection were assigned half of the value of the lowest standard value. All samples were measured in duplicate and the absorbance was measured at 450nm for cytokines on a microtitre plate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA). The coefficient of variance was ≤10% for the assay.

6.2.6 Splenocyte Phospholipid Fatty Acid Composition

A modified Folch method was used to extract lipids from isolated splenocytes as previously described (38). Individual phospholipids were separated on thin layer chromatography plates (HPK silica gel 60nm 10x10cm; Fisher Scientific, Edmonton, AB, Canada) (39) and visualized with 8-anilino-1-naphthalenesulfonic acid under ultraviolet light against the appropriate standards. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) methyl esters were prepared from the scraped silica bands (38) and separated by automated gas liquid chromatography (Varian 3800, Varian Instruments, Mississauga, ON, Canada) using a 100m *CP*-Sil 88 fused capillary column (Varian Instruments, Mississauga, ON, Canada) as described elsewhere (40).

6.2.7 Statistics

Statistical analysis was conducted by one way ANOVA using the SAS software statistical package (Version 9.1, SAS Institute, Cary, NC). All data was reported as mean ± standard error of the mean (SEM). Significant differences among groups were determined by Duncan's multiple range test ($P<0.05$) and all non-parametric data was log-transformed prior to running statistical analyses.

6.3 Results

6.3.1 Feed Intake, Body Weight and Spleen Characteristics

Obese rats had higher feed intake (3855 ± 55 g vs 2360 ± 51 g, $p<0.05$), final body weight (669 ± 8 g vs 375 ± 10 g, $p<0.05$), and spleen weight (1174 ± 82 mg vs 840 ± 82 mg, $p<0.05$) than lean rats. When adjusted for body weight (bwt), spleens of obese rats weighed less than lean rats (1.7 ± 0.1 mg/g bwt vs. 2.3 ± 0.3 mg/g bwt, $p<0.05$), but the concentration of splenocytes per gram of spleen did not differ between lean and obese rats ($2.2 \pm 0.3 \times 10^7$ cells vs. $2.2 \pm 0.2 \times 10^7$ cells, $p<0.05$). Obese rats fed LFO (584 ± 12 g) or HFO (552 ± 10 g) diet had lower final body weights than the obese rats fed the control diet (669 ± 8 g, $p<0.05$). Feed intake was lower for LFO (3625 ± 62 mg) compared to obese Ctl, but higher than HFO fed rats (3325 ± 63 mg) ($P<0.05$). Feeding FO did not alter spleen weight (LFO, 1930 ± 130 mg, or HFO, 1910 ± 75 mg) or concentration of immune cells (LFO, $1.9 \pm 0.2 \times 10^7$, or HFO, $1.7 \pm 0.2 \times 10^7$) in the spleen.

6.3.2 Splenocyte Phospholipid Fatty Acid Composition

PC: Compared to lean rats, obese rats fed the Ctl diet had a higher ($P<0.05$, Table 6.3) proportion of 16:0, 18:1(n-9), 18:1(n-7), total MUFA and a lower proportion of 18:0, (n-6) PUFA and (n-6):(n-3) PUFA in PC fraction of splenocytes ($P<0.05$). Rats fed either FO diet had a higher percentage of 18:1(n-9), 20:5(n-3), 22:5(n-3), 22:6(n-3), total MUFA and total (n-3) PUFA and a lower proportion of 20:4(n-6), total (n-6) PUFA and (n-6):(n-3) PUFA compared to obese rats fed the Ctl diet ($P<0.05$. Table 6.3). Relative to LFO-fed rats, HFO-fed rats had a higher percentage of 18:1(n-9), 20:5(n-3) and total (n-3) PUFA and lower 18:2(n-6), 20:4(n-6), total MUFA, PUFA and (n-6) PUFA ($P<0.05$, Table 6.3).

Table 6.3. Fatty acid composition of PC splenocytes from lean and obese Ctl rats and LFO and HFO-fed rats

	Ln Ctl	Obese Ctl	LFO	HFO
	g/100g			
16:0	29 ± 0^{b}	39 ± 1^{a}	40 ± 2^{a}	40 ± 1^{a}
18:0	23 ± 0^{a}	14 ± 0^{b}	13 ± 0^{b}	13 ± 0^{b}
18:1(n-9)	7.9 ± 0.1^{d}	9.1 ± 0.1^{c}	11 ± 0^{b}	12 ± 0^{a}
18:1(n-7)	1.7 ± 0.1^{b}	3.3 ± 0.1^{a}	3.2 ± 0.1^{a}	3.4 ± 0.1^{a}
18:2(n-6)	11 ± 0.6^{b}	9.8 ± 0.4^{b}	13 ± 0.3^{a}	9.6 ± 0.3^{b}
18:3(n-3)	0.55 ± 0.04	0.54 ± 0.04	0.54 ± 0.09	0.54 ± 0.06
20:4(n-6)	17 ± 1^{a}	15 ± 1^{a}	6.6 ± 0.4^{b}	4.2 ± 0.3^{c}
20:5(n-3)	0.05 ± 0.01^{c}	0.14 ± 0.03^{c}	2.5 ± 0.2^{b}	4.1 ± 0.3^{a}
22:5(n-3)	0.31 ± 0.02^{b}	0.53 ± 0.08^{b}	2.0 ± 0.2^{a}	2.3 ± 2^{a}
22:6(n-3)	0.39 ± 0.0^{4b}	0.63 ± 0.10^{b}	1.4 ± 0.2^{a}	1.5 ± 0.1^{a}
MUFA	10 ± 0^{d}	14 ± 0^{c}	16 ± 0^{b}	19 ± 0^{a}
SFA	54 ± 1	55 ± 1	54 ± 1	55 ± 1
PUFA	32 ± 2^{a}	29 ± 1^{a}	28 ± 1^{a}	24 ± 1^{b}
PUFA:SFA	0.60 ± 0.04^{a}	0.53 ± 0.03^{ab}	0.52 ± 0.04^{ab}	0.44 ± 0.02^{b}
(n-6) PUFA	31 ± 2^{a}	27 ± 1^{b}	22 ± 1^{c}	15 ± 1^{d}
(n-3) PUFA	1.3 ± 0.1^{c}	1.9 ± 0.2^{c}	6.4 ± 0.6^{b}	8.9 ± 0.4^{a}
(n-6):(n-3) PUFA	24 ± 1^{a}	15 ± 1^{b}	3.4 ± 0.2^{c}	1.8 ± 0.1^{c}

Data represent mean ± SEM; n=5/group. Means within the same row that do not share a common letter are significantly different ($p<0.05$). The relative proportions of fatty acids from 12:0 to 24:1(n-9) were measured in whole splenocyte membrane phospholpids, but only the major fatty acids were reported. Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

PE: Compared to lean rats, obese rats fed the Ctl diet had a higher proportion of 16:0, 18:1(n-7) and a lower proportion of 18:0, 22:5(n-6) , total (n-6) PUFA and (n-6):(n-3) PUFA in the PE fraction of splenocyte membranes ($P<0.05$, Table 6.4). Rats fed either FO diet had a higher percentage of 18:1(n-9), 20:5(n-3), 22:5(n-3), 22:6(n-3) and total (n-3) PUFA and a lower proportion of 18:0, 20:4(n-6), 22:4(n-6), 22:5(n-6), total (n-6) PUFA and (n-6):(n-3) PUFA ratio compared to Obese Ctl-fed rats ($p<0.05$) ($P<0.05$, Table 6.4). In comparison to obese rats fed the LFO diet, HFO-diet fed rats had a lower proportion of 18:2(n-6), 20:4(n-6) and (n-6) PUFA and a higher percentage of 20:5(n-3) and total (n-3) PUFA ($P<0.05$, Table 6.4).

Table 6.4. Fatty acid composition of PE splenocytes from lean and obese Ctl rats and LFO and HFO-fed rats

	Lean Ctl	Obese Ctl	LFO	HFO
		g/100g		
16:0	6.3 ± 0.8^{b}	12 ± 1^{a}	11 ± 1^{a}	12 ± 1^{a}
18:0	37 ± 4^{a}	29 ± 1^{ab}	25 ± 1^{b}	27 ± 1^{b}
18:1(n-9)	3.5 ± 0.4^{b}	3.6 ± 1^{b}	5.3 ± 0.5^{a}	5.6 ± 0.1^{a}
18:1(n-7)	0.52 ± 0.10^{b}	1.0 ± 0.1^{a}	1.0 ± 0.1^{a}	1.0 ± 0.0^{a}
18:2(n-6)	2.8 ± 0.4^{b}	2.7 ± 0.2^{b}	4.5 ± 0.6^{a}	3.4 ± 0.1^{b}
18:3(n-3)	0.16 ± 0.01^{c}	0.21 ± 0.01^{bc}	0.23 ± 0.03^{ab}	0.28 ± 0.02^{a}
20:4(n-6)	32 ± 2^{a}	31 ± 2^{a}	18 ± 1^{b}	12 ± 0^{c}
20:5(n-3)	0.12 ± 0.00^{c}	0.32 ± 0.09^{c}	6.4 ± 1^{b}	10 ± 0^{a}
22:4(n-6)	5.9 ± 0.7^{a}	6.1 ± 0.8^{a}	0.66 ± 0.05^{b}	0.41 ± 0.02^{b}
22:5(n-6)	0.82 ± 0.14^{a}	0.55 ± 0.04^{b}	0.17 ± 0.01^{c}	0.22 ± 0.01^{c}
22:5(n-3)	1.5 ± 0.2^{b}	3.1 ± 0.5^{b}	10 ± 0^{a}	12 ± 1^{a}
22:6(n-3)	1.5 ± 0.2^{b}	2.7 ± 0.5^{b}	6.2 ± 0.1^{a}	6.9 ± 0.3^{a}
MUFA	6.6 ± 1.2^{b}	8.8 ± 1.2^{ab}	11 ± 1^{a}	11 ± 0^{a}
SFA	45 ± 3^{a}	43 ± 2^{ab}	38 ± 2^{b}	40 ± 1^{ab}
PUFA	42 ± 2	41 ± 2	43 ± 2	$43 \pm .1$
PUFA:SFA	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.0
(n-6) PUFA	42 ± 2^{a}	37 ± 1^{b}	24 ± 1^{c}	17 ± 0^{d}
(n-3) PUFA	3.5 ± 0.4^{c}	6.1 ± 1.0^{c}	23 ± 1^{b}	29 ± 1^{a}
(n-6):(n-3) PUFA	12 ± 1^{a}	6.4 ± 0.8^{b}	1.0 ± 0.0^{c}	0.60 ± 0.02^{c}

Data represent mean ± SEM; n=5/group. Means within the same row that do not share a common letter are significantly different ($p<0.05$). The relative proportions of fatty acids from 12:0 to 24:1(n-9) were measured in whole splenocyte phospholipid membrane, but only the major fatty acids were reported. Abbreviations used: ND, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; (n-6) PUFA, sum of (n-6) polyunsaturated fatty acids; (n-3) PUFA, sum of (n-3) polyunsaturated fatty acids.

6.3.3 Phenotypes: Freshly isolated splenocytes

Obese rats fed the Ctl diet had a higher proportion of $CD4^+CD25^+$ (T helper cells expressing the IL-2 receptor) and $CD4^+$ splenocytes that expressed CD25 and CD28 (co-stimulatory molecule) relative to the lean Ctl group ($P<0.05$, Table 6.5). Obese Ctl rats also had a significantly lower proportion of $CD11b/c^+OX6^+$ (non-B-cell antigen presenting cells expressing the major histocompatibility complex (MHC) class II) compared to lean Ctl rats ($P<0.05$). Furthermore, obese Ctl rats had a higher proportion of $CD11b/c^+$ splenocytes that expressed $OX6^+/CD80^+$ (activated non-B-cell antigen presenting cells).

Obese rats fed either FO diet had a lower proportion of $CD8^+CD25^+$ (cytotoxic T-cells expressing IL-2 receptor), $CD4^+CD25^+$ and a lower percentage of $CD4^+$ or $CD8^+$ cells expressing CD25 and CD28 ($P<0.05$, Table 6.5). Obese rats fed the HFO diet had a lower proportion of $CD3^+$ cells (T-cells), which affected mainly the $CD8^+$ cells as there was no difference in the percentage of $CD3^+CD4^+$. Compared to obese Ctl rats, obese FO-fed rats had a lower proportion of $CD11b/c^+CD80^+$, $OX6^+CD80^+$ and a lower percentage of $CD11b/c^+$ cells that expressed CD80 and OX6. The HFO group also had a lower proportion of $CD8^+CD28^+$ splenocytes relative to the obese Ctl group ($P<0.05$, Table 6.5). In comparison to the LFO group, the HFO group had a higher proportion of $CD4^+$ and $CD4^+CD28^+$ and a lower proportion of $CD3^+$, $CD8^+$, $CD3^+CD8^+$, $CD4^+CD25^+$, and a lower percentage of $CD4^+$ cells expressing CD28 and CD25 ($P<0.5$, Table 6.5).

Table 6.5. Freshly isolated splenocyte phenotypes of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA-*cp* rats fed the Ctl, LFO or HFO diet.

	Lean Ctl	Obese Ctl	LFO	HFO
$CD3^+$	47 ± 1^a	48 ± 1^a	47 ± 1^a	43 ± 1^b
$CD8^+$	13 ± 1^a	13 ± 0^a	13 ± 1^a	11 ± 0^b
$CD4^+$	45 ± 1^{ab}	42 ± 2^b	43 ± 2^b	48 ± 1^a
$CD3^+CD8^+$	10 ± 0^a	11 ± 0^a	10 ± 1^a	8.8 ± 0.3^b
$CD3^+CD4^+$	36 ± 1^a	35 ± 1^a	35 ± 1^a	34 ± 1^a
$CD8^+CD28$	11 ± 0.5^a	11 ± 0.4^{ab}	9.5 ± 0.3^{bc}	8.3 ± 0.3^c
$CD8^+CD25^+$	0.77 ± 0.09^a	0.86 ± 0.12^a	0.43 ± 0.07^b	0.37 ± 0.08^b
$CD8^+CD25^+CD28^+$	5.8 ± 0.4^a	6.4 ± 0.7^a	3.7 ± 0.4^b	3.8 ± 0.7^b
$CD4^+CD28^+$	43 ± 1^{ab}	44 ± 1^{ab}	42 ± 2^b	47 ± 1^a
$CD4^+CD25^+$	4.4 ± 0.3^b	6.3 ± 0.4^a	3.2 ± 0.3^c	2.0 ± 0.2^d
$CD4^+CD28^+CD25^+$	9.9 ± 0.6^b	13 ± 0.9^a	6.5 ± 0.7^c	4.3 ± 0.5^d
$OX6^+CD11b/c^+$	11 ± 0.4^a	8.0 ± 0.3^b	8.9 ± 0.5^b	7.9 ± 0.5^b
$OX6^+CD80^+$	5.4 ± 0.3^a	5.9 ± 0.3^a	4.1 ± 0.2^b	4.5 ± 0.3^b
$CD11b/c^+CD80^+$	4.4 ± 0.6^{ab}	4.7 ± 0.3^a	3.1 ± 0.3^c	3.6 ± 0.4^{bc}
$CD11b/c^+CD80-$	13 ± 1^a	9.7 ± 0.9^b	13 ± 1^a	11 ± 0.5^b
$CD11b/c^+OX6^+CD80^+$	22 ± 2^b	29 ± 3^a	18 ± 0^b	20 ± 2^b

Data represent mean ± SEM; n=8/group. Values are a proportion of the total gated cells as determined by immunofluorescence. Means within the same row that do not share a common letter are significantly different ($p<0.05$). ND, not determined.

6.3.4 Phenotypes: ConA-Stimulated Splenocytes

Splenocyte phenotypes were also determined following stimulation with a polyclonal T-cell mitogen. Compared to lean Ctl rats, obese Ctl rats had a higher proportion of $OX6^+CD80^+$ cells (antigen presenting cells expressing co-stimulatory molecule) ($P<0.05$, Table 6.6). There were no differences in T-cells or subsets or any other immune cell measured between lean and obese Ctl rats.

Obese rats fed FO had a lower proportion of $CD8^+CD28^+$ (cytotoxic T-cells expressing co-stimulatory molecule) ($P<0.05$, Table 6.6). Obese rats fed the LFO diet had a higher percentage of $OX6^+CD80^+$ (activated antigen presenting cells). The HFO-fed group had a lower proportion of T-cells ($CD3^+$), which was due to a lower proportion of $CD8^+$ T-cells, including activated $CD8^+$ T-cells ($CD8^+CD28^+CD25^+$) ($P<0.05$, Table6.6). Obese rats fed the HFO di*et al*so had a lower percentage of $CD11b/c^+OX6^+$ (non-B-cells antigen presenting cells) and $OX6^+CD80^+$ (activated antigen presenting cells) and $OX12^+$ (B-cells) ($P<0.05$, Table 6.6).

Table 6.6. Immune cell phenotypes of ConA-stimulated splenocytes of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA-*cp* rats fed the Ctl, LFO or HFO diet.

	Lean Ctl	Obese Ctl	LFO	HFO
$CD3^+$	48 ± 1^a	48 ± 3^a	49 ± 1^a	43 ± 1^b
$CD3^+CD8^+$	9.0 ± 0.33^a	8.9 ± 0.49^a	9.1 ± 0.24^a	7.5 ± 0.46^b
$CD3^+CD4^+$	34 ± 0.33	34 ± 2.2	34 ± 0.7	34 ± 1.0
$CD8^+CD28^+$	11 ± 0.4^{ab}	12 ± 0.5^a	10 ± 0.4^b	9.9 ± 0.7^b
$CD8^+CD28^+CD25^+$	6.2 ± 0.25^a	5.8 ± 0.69^a	4.9 ± 0.56^a	3.2 ± 0.37^b
$CD4^+CD28^+CD25^+$	16 ± 1.5	15 ± 2.0	18 ± 0.8	16 ± 0.4
$CD11b/c^+OX6^+$	8.0 ± 0.77^a	8.0 ± 1.7^a	9.1 ± 0.2^a	4.3 ± 0.2^b
$CD11b/c^+OX6^+CD80^+$	3.5 ± 0.38^a	3.0 ± 0.50^a	2.3 ± 0.18^a	0.93 ± 0.08^b
$OX6^+CD80^+$	3.5 ± 0.4^a	$2.2 \pm 0.8b$	3.5 ± 0.4^a	1.3 ± 0.1^c
$OX12^+$	41 ± 2.5^a	36 ± 3.1^a	40 ± 1.9^a	29 ± 0.89^b

Data represent mean ± SEM; n=8/group. Values are a proportion of the total gated cells as determined by immunofluorescence. Means within the same row that do not share a common letter are significantly different ($p<0.05$). The proportion of $CD8^+CD25^+$ (4.9 ± 0.9, N=32), $CD4^+CD28^+$ (34 ± 1, N=32), $CD4^+CD25^+$ (22 ± 1, N=32), and $CD11b/c^+CD80^+$ (2.9 ± 0.5, N=32).

6.3.5 Cytokine Production

Compared to lean rats, mitogen-stimulated splenocytes of obese rats fed the Ctl diet produced significantly more IL-2 (ConA) and IFN-γ (ConA and LPS) ($P<0.05$, Table 6.7). Obese rats fed the HFO diet produced more ConA-stimulated IL-2 production compared to the obese Ctl and LFO group ($P<0.05$, Table 6.7). Production of TNF-α from LPS-stimulated splenocytes was also lower in the HFO group ($P<0.05$, Table 6.7). ConA-stimulated IL-6 and IL-1β production and LPS-stimulated IL-10 and IL-1β production did not differ between lean and obese rats ($P<0.05$, Table 6.7). Feeding FO to obese JCR:LA-*cp* rats did not affect ConA or LPS-stimulated IL-1β, IL-10 or IL-6 production compared to the obese Ctl group.

Table 6.7. Splenocyte mitogen-stimulated cytokine production of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA-*cp* rats fed the Ctl, LFO or HFO diet

	Lean Ctl	Obese Ctl	LFO	HFO
	(pg/ml)			
ConA				
IL-2	1115 ± 224^{c}	2017 ± 132^{b}	2096 ± 171^{b}	3067 ± 352^{a}
IL-1β	9.1 ± 2.0^{b}	25.7 ± 7.5^{ab}	28.2 ± 5.2^{a}	30.4 ± 8.8^{a}
IL-10	502 ± 46^{b}	581 ± 99^{ab}	845 ± 220^{ab}	830 ± 128^{a}
IL-6	507 ± 160^{a}	227 ± 31^{a}	533 ± 185^{a}	345 ± 77^{a}
IFN-γ	117 ± 46^{b}	299 ± 71^{a}	160 ± 43^{ab}	247 ± 42^{ab}
LPS				
IL-1β	71 ± 4.5^{a}	77 ± 12^{a}	105 ± 13^{a}	88 ± 16^{a}
TNF-α	354 ± 50^{a}	338 ± 61^{a}	393 ± 27^{a}	176 ± 38^{b}
IL-10	1146 ± 68^{a}	1193 ± 80^{a}	1266 ± 95^{a}	1245 ± 88^{a}
IFN-γ	16 ± 2.4^{b}	98 ± 24^{a}	70 ± 22^{a}	82 ± 25^{a}

Data represent mean ± SEM; n=8/group. Means within the same row that do not share a common letter are significantly different ($p<0.05$).

6.3.6 PKC-θ in ConA-stimulated Splenocytes

The levels of PKC-θ in the cytoplasm of ConA-stimulated splenocytes were higher in obese Ctl rats relative to lean Ctl rats ($P<0.05$, Fig.6.1). However, levels of phospho-PKC-θ did not differ between lean and obese rats. Feeding the HFO diet to obese rats lowered PKC-θ to levels similar to lean Ctl rats ($P<0.05$, Fig.6.1) and levels of phospho-PKC-θ were lower with HFO feeding compared to obese Ctl rats ($P<0.05$, Fig.6.1). Obese rats fed the LFO diet had lower phospho-PKC-θ levels compared to obese Ctl rats ($P<0.05$, Fig. 6.1), but total PKC-θ levels did not differ significantly from any other group.

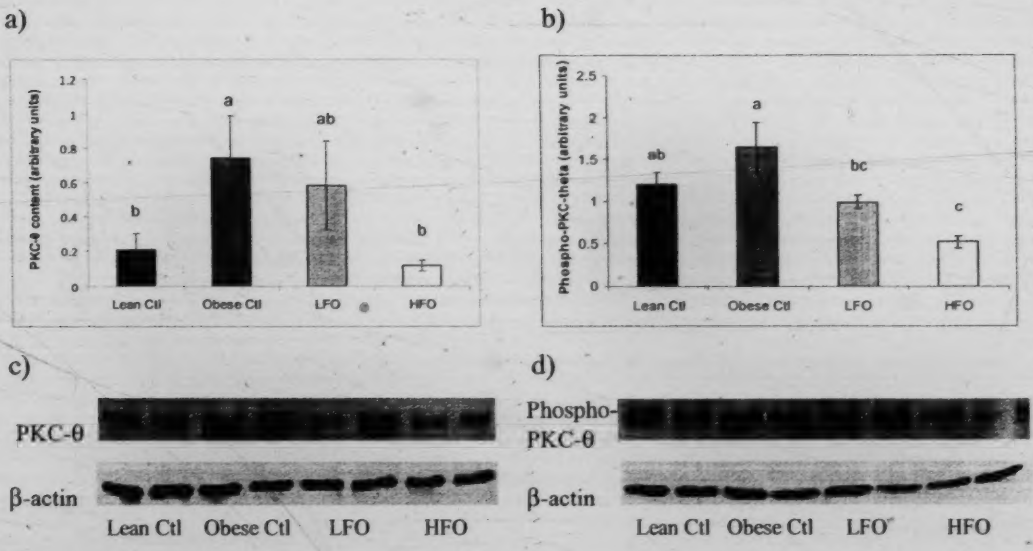

Figure 6.1. a) Relative total PKC-θ and b) phospho-PKC-θ levels in ConA-stimulated (18h) splenocytes. Bars represent mean ± SEM; n=8/group. Bars not sharing a common letter are significantly different ($P<0.05$). Representative Western blots for c) total PKC-θ and d) phospho-PKC-θ.

6.4 Discussion

This study revealed that relative to lean rats, splenocytes of the obese, leptin receptor deficient JCR:LA-*cp* rat produced nearly 2-fold greater IL-2 and 2.5-fold greater IFN-γ in response to a polyclonal T-cell mitogen, indicating a skewed Th1 cytokine response. This is contrary to studies conducted in other rodent models of obesity, which have reported that the proliferative response of immune cells and the ability to produce IL-2 in response to T-cell mitogens is impaired in diet-induced obese rodents (20-24), *fa/fa* Zucker rats (15-18) and obese offspring of diabetic dams (19). The higher IL-2 and IFN-γ production identified in obese JCR:LA-*cp* rats is intriguing in light of the evidence that suggests that leptin signalling via the long form of the leptin receptor is required for adequate IL-2 secretion and the promotion of IFN-γ production (41, 42). Splenocytes from this obese model secreted more IL-2 in the absence of a functional leptin receptor suggesting that there are additional contributing factors. There were no differences in the relative proportion of T-helper cell subsets between lean and obese Ctl rats that might explain the higher *ex vivo* IL-2 production. Furthermore, it is not likely that the lower (n-6):(n-3) PUFA ratio of splenocyte PL could account for the differences in cytokine

production. Studies carried out in other inflammatory disease states suggest that lowering the (n-6):(n-3) PUFA ratio in immune cell phospholipids is associated with reducedinflammation and disease progression, possibly via reductions in Th1 cytokine responses (as reviewed by (43)). In the present study, despite the significantly lower (n-6):(n-3) PUFA ratio in both PE and PC phospholipids, splenocytes from obese Ctl rats produced more IL-2 and IFN-γ than cells from lean rats. This lower (n-6):(n-3) PUFA ratio has also been reported in other obese rodents (15, 19, 44, 45) and confirms that the altered hepatic lipid metabolism (46) reported in obesity affects immune cell fatty acid composition. Additionally, the percentage of activated T-cells (those expressing CD25 or CD28) in ConA-stimulated splenocytes did not differ between lean and obese rats suggesting that the increased Th1 cytokine response was due to modifications in intracellular signalling rather than a change in the proportion of activated T cells. To explore this, we chose to examine the major signalling pathway from the T-cell receptor (TCR) leading to IL-2 production. PKC-θ is a Ca^{+2}-independent serine/threonine kinase expressed in T-lymphocytes, platelets and muscle tissue (as reviewed by (32)). It appears to be essential for IL-2 production based on *in vitro* studies in PKC-θ deficient mice (33) and can activate the transcription factors NF-κB, NFAT and AP-1 c-Fos that lead to IL-2 transcription (as reviewed by (32)). In the present study, obese Ctl rats had significantly higher concentrations of total PKC-θ relative to lean Ctl rats, suggesting that this may be at least partly contribute to the higher IL-2 production. Although there was a trend towards a higher level of the activated form, phosphorylated PKC-θ, in cells from obese Ctl JCR:LA-*cp* rats, this difference did not reach statistical significance. Prior to this study, the role of PKC-θ in T-lymphocyte function in obesity had not been examined. However, researchers have reported greater expression (47) and activity (48) of PKC-θ in muscle tissue of obese subjects and have implicated this isoform in inducing insulin resistance by serine phosphorylation of the insulin receptor substrate-1 (49). While the increased level of total PKC-θ is suggestive of altered T-cell function, the absence of a significant difference in the activated form questions this hypothesis. Thus, further investigation in isolated T-cells is necessary to establish the mechanistic pathways involved in eliciting the higher IL-2 response.

As hypothesized, feeding either the LFO or HFO diet further lowered the (n-6):(n-3) PUFA ratio and increased the proportion of EPA and DHA into PE and PC fractions of the splenocyte membrane. Feeding FO to obese rats lowered ConA-stimulated IFN-γ to levels not different from lean rats, suggesting that long chain (n-3) PUFA can normalize the Th1 cytokine response. It could be hypothesized that the lower weight gain observed in the FO-fed rats may have affected IFN-γ production. However, HFO rats weighed less than LFO rats, and there was no difference in IFN-γ production, suggesting that the difference in body mass was not the explanation for the lower IFN-γ. Obese rats fed the HFO diet produced even greater amounts of IL-2 as compared to obese Ctl rats. This is a notable finding given that previous work has consistently reported that mitogen-stimulated IL-2 production is reduced in healthy rodents fed FO or long chain (n-3) PUFA (34, 50-52). Few researchers have investigated the impact of dietary (n-3) PUFA on immune health in obesity. Khan *et al* (2006) (31) reported that IFN-γ and IL-2 mRNA levels in spleen were higher in obese offspring of diabetic rats and that feeding long chain (n-3) PUFA (~2.1%w/w) normalized these levels. However, the lower IL-2 levels differed from our results, but may be due to the age of the rats used and/or the fact that only mRNA levels on whole spleen tissue were measured. In addition, we observed no significant differences in the proportion of activated T-helper cells 18 h post ConA stimulation that might explain the 2-fold increased IL-2 concentrations. Thus, regardless of a non-functional leptin receptor and a high level of EPA and DHA in phospholipids, splenocytes produced more of a T-cell proliferative cytokine in the obese JCR:LA-*cp* rat, suggesting other mechanisms are involved.

Hence, we determined the level of PKC-θ in ConA stimulated splenocytes of obese rats fed FO. As compared to obese rats fed the Ctl diet, rats fed the HFO diet had significantly lower levels of PKC-θ and phosphorylated PKC-θ, which would not be predicted, based on the higher levels of IL-2 produced by stimulated splenocytes from HFO-fed rats. Our results suggest that the HFO diet may activate a protein downstream of PKC-θ or another pathway leading to IL-2 synthesis. The LFO diet affected PKC-θ, particularly total levels, less than the HFO diet. This is the first study to examine the effects of dietary EPA and DHA on PKC-θ levels in obese rats; only one other group has reported the effects in healthy mice. Fan *et al* (2004) (34) observed that FO-

supplemented (4%w/w) mice had reduced localization of PKC-θ to lipid rafts of anti-CD3/anti-CD28 stimulated $CD4^+$ T-cells. Although not stated in Fan *et al*'s study, it is possible that the total amount of PKC-θ was lower than the control, as observed in our study, which may account for the decreased localization of PKC-θ to lipid rafts. Regardless, our study and Fan *et al*'s (34) demonstrate that PKC-θ is affected by dietary EPA and DHA. Clearly, the 3-fold decrease in phosphorylated PKC-θ levels relative to the obese Ctl group does not explain the 2-fold increase in IL-2 secretion observed in obese JCR:LA-*cp* rats fed the HFO diet and warrants further investigation. We have previously reported that dietary EPA and DHA can modify the fatty acid composition of lipid rafts in obese JCR:LA-*cp* rats. Hence, future research should examine how this affects localization of PKC-θ to lipid rafts {Ruth2008A}.

6.5 Conclusion

This study demonstrates that obese JCR:LA-*cp* rats produced more Th1 cytokines in response to a polyclonal T-cell mitogen and have altered fatty acid composition of splenocyte PE and PC phospholipids. Moreover, this is the first study to show that the higher IL-2 production observed in obese JCR:LA-*cp* rats may be partly due to higher cytoplasmic concentrations of PKC-θ, a key signalling molecule leading to IL-2 synthesis. Early intervention and long term dietary intake of EPA and DHA normalized the production of IFN-γ, a Th1 cytokine. Moreover, the HFO diet further enhanced production of ConA-stimulated IL-2 through mechanisms independent of cytoplasmic levels of total and phosphorylated PKC-θ and additional experiments are necessary to identify the cellular mechanisms.

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7 COMPARISON OF IMMUNE FUNCTION IN THREE RODENT MODELS OF OBESITY: DIET-INDUCED, JCR:LA-*CP* AND ZUCKER RATS

7.1 INTRODUCTION

Due to the alarming increase in the incidence of obesity, there is growing interest in the metabolic consequences of accumulating excess fat mass. Several chronic diseases with underlying sub-clinical inflammation including cardiovascular disease, type 2 diabetes and cancer are more prevalent in the overweight population, suggesting that inflammation may be the driving factor in disease progression and pathology (1). Although there is considerable evidence that chronic low-grade inflammation is associated with the obese state, the aetiology of this inflammation is not known. Adipocytes and infiltrated immune cells have been implicated as major contributors to the milieu of circulating inflammatory markers; however, the exact roles of these cells and the immune system as a whole have not been delineated, nor have any underlying mechanistic abnormalities. Although several rodent models of obesity have been utilized to examine the effects of obesity on immune function, there is no agreement on what is the best model, nor has there been a systematic comparison of the immunological changes in various models. This chapter describes and compares the immunological changes in three well-established rodent models of obesity and insulin resistance: the *fa/fa* Zucker rat, the JCR:LA-*cp* rat and a diet-induced obese (DIO) rat.

The Zucker *fa/fa* rat is a monogeneic model of obesity that expresses a dysfunctional leptin receptor that severely limits its ability to respond to leptin (2), a condition that is extremely rare in humans and has been identified in only a few individuals (3). However, many of the metabolic changes seen in the Zucker *fa/fa* rat are observed in human obesity (4, 5). Hyperphagia leading to excessive weight gain develops in this model by 5-6 weeks of age and is accompanied by other metabolic defects, such as dyslipidemia and hyperinsulinemia. A limited number of studies have been conducted on immune function in this animal model. Abnormalities in the innate immune system have been identified in the *fa/fa* Zucker rat including an impaired capacity to kill yeast, despite normal phagocytic function (6, 7). Additionally, T-cell lymphopenia affecting both the $CD4^+$ and the $CD8^+$ T cells (8) and a decreased ability of lymphocytes to respond *in vitro* to mitogen stimulation have been reported (9, 10). Little

is known about the effect of obesity on mitogen-stimulated cytokine, immunoglobulin production or immune cell types (beyond the relative proportion of $CD4^+$ and $CD8^+$ cells). Although these studies are suggestive of immune dysfunction, there is currently no animal model of obesity with identified chronic inflammation or clearly characterized T cell dysfunction that could be used to study immune abnormalities observed in human obesity.

Similar to the *fa/fa* Zucker rat, the JCR:LA-*cp* rat is a genetic model of obesity that expresses a dysfunctional leptin receptor. However, unlike the *fa/fa* Zucker rat, which has some residual leptin-receptor activity, the obese JCR:LA-*cp* rat has no known leptin-mediated signal through the long form of the leptin receptor (Ob-R). Rats that are homozygous for the autosomal recessive *cp* gene (*cp/cp*) are obese and insulin resistant, have dyslipidemia and develop atherosclerosis (11-13). Consequently, this model has been used extensively to study the impact of metabolic syndrome on various parameters of cardiovascular disease. Prior to our investigations, there had been no published studies characterizing immune function in this animal mode.

Another common rodent model employed to determine the impact of obesity on immune function is the DIO mouse or rat. The diets used to induce excessive weight gain in immunology studies, include high fat (35-70% of calories), high sucrose/high fat or a mixture of highly saturated foods. Although inconsistencies reside in the literature, impairments in both T-cell function (14-18) and inflammatory cytokine responses (19) have been identified in DIO rodents. In order to adequately compare the DIO model to the JCR:LA-*cp* rat and *fa/fa* Zucker rats, we used male Wistar rats fed a high fat (HF) diet (60% energy) and examined the response of splenocytes to mitogen stimulation under similar experimental conditions used for the genetic models of obesity.

7.2 Materials and Methods

fa/fa Zucker Rat: Refer to Chapter 3 (page 69-73) for methods. Refer to Table 7.1 and 7.2 for diet and fatty acid composition of experimental diet.

JCR:LA-*cp*: Refer to Chapter 4 (page 96-101) for methods. Refer to Table 7.1 and 7.2 for diet and fatty acid composition of experimental diet.

7.2.1 DIO Rats

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Agricultural, Life and Environmental Sciences Animal Ethics Committee at the University of Alberta. Male Wistar rats (n=16, Charles River, Wilmington, MA) were housed 2 per cage, with 12/12-hour light/ dark cycle and had free access to water and were fed standard rat chow, *ad libitum*. Rats were acclimated for one week and then randomly assigned to either the chow diet group (n=8) or high fat diet group (n=8). Refer to Table 7.1 and 7.2 for the diet and fatty acid composition of the standard chow (5001 rodent diet, LabDiet, Canadian Lab Diets, Leduc, AB, Canada) and HF diet. The micronutrient levels are adequate for the rats in each group, although the density likely differs on an energy basis. Animals were anesthesized with sodium pentabarbitol (5mg/100g body weight) and spleens were removed aseptically.

Table 7.1. Composition of experimental diets

	DIO	JCR:LA-cp	Zucker
		g/kg	
Casein	254	270	-
Egg White	-	-	213
Corn Starch	169	212	363
Dextrose	-	215	-
Maltodextrin	-	-	132
Sucrose	85	-	100
Bran	51	-	-
Cellulose	-	79	50
Vitamin Mix	11.7	9.4	35
Mineral Mix	67	48	10
Choline Chloride	1.3	2.7	2.5
Methionine	3	2.5	-
Gelatin	19	-	-
Inositol	-	6.2	-
Biotin Mix	-	-	10

Nutrient composition varies among the three groups, but meets the nutrient needs of each rat model.

Table 7.2. Fatty acid composition of dietary experimental oils.

	Chow[1]	HF Diet[2]	JCR:LA-*cp*[3]	Zucker[4]
MUFA	28	39	10	23
SFA	27	30	65	16
PUFA	25	27	25	58
PUFA:SFA	0.9	0.9	0.4	3.6
(n-6) PUFA	21	26	23	51
(n-3) PUFA	3.3	1.1	1.2	7
(n-6):(n-3) PUFA	6.4	24	19	7.3

Values represent percentage of total fat. The total amount of fat added (w/w): [1] 5.7% (LabDiet 5001), [2]34%, [3]15%, [4]8.5%. MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

7.2.2 Isolation of Splenocytes and Primary Culture Conditions

The spleens were weighed and placed in sterile 0.5% w/v bovine serum albumin in Krebs-Ringer HEPES buffer (pH 7.4) and isolated as we have previously described in detail (20). Isolated splenocytes were resuspended in complete culture media (RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic-antibiotic solution, HEPES (25 mmol/L), and 2-mercaptoethanol (2.5 mmol/L)) and counted on a haemacytometer (Fisher Scientific, Edmonton). Splenocytes ($1.25x10^6$ cells/L) were resuspended in the culture media described above and incubated in 4 mL sterile polystyrene tubes in a humidified atmosphere at 37°C in the presence of 5% v/v CO_2. Splenocytes were cultured without mitogen (unstimulated cells) or with ConA (2.5mg/L) or LPS (1mg/L) as we have previously described (21). After 48 h of culture, the supernatant was removed and stored at -80° C until cytokine assays were performed.

7.2.3 Phenotype Analysis

Immune cell subsets in freshly isolated splenocytes were identified by one, two or three colour direct immunofluorescence assay as we have previously described (22). The following pre-labelled mAbs were used: CD3 and CD11b/c (FITC-labelled); CD4 (APC-labelled), CD8 and CD80 (RPE-CY5), and OX12, OX6, and CD25 (PE-labelled). All antibodies were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada) except CD4, CD80 and OX12, which were purchased from Serotec (Cedarlane Laboratories Ltd, Hornby, ON, Canada). After final wash, plates were aspirated and 200

uL of cell fix (1% w/v paraformaldehyde) was added to each well. The proportion of cells that were positive for each marker was determined by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA) according to the relative fluorescence intensity using CellQuest software (Becton-Dickinson).

7.2.4 Cytokine Production

All of the following assays were performed according to the manufacturer's instructions. The cultured cell supernatants of unstimulated, ConA and LPS-stimulated splenocytes were used to determine IL-1β and TNF-α (detection limit: 31.2-2000pg/mL), IL-2 (23.4-1500 pg/mL), IL-4 (1.6-100 pg/mL), IL-6 (78-5000 pg/mL), IL-10 (15.6-1000 pg/mL) and IFN-γ (31.25-2000 pg/mL) levels with OptEIA anti-rat enzyme-linked immunoasorbant assay (ELISA) (BD Biosciences, PharMingen, Mississauga, ON, Canada). Values below the range of detection were assigned half of the value of the lowest standard value. All samples were measured in duplicate and the absorbance for cytokine assays was measured at 450nm on a microtitre plate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA). If the coefficient of variance exceeded 10% for duplicate samples, the samples were re-run. The average of the duplicate data with a coefficient of variance of ≤10% was used for statistical analysis.

7.2.5 Statistics

Statistical analysis was conducted by one way ANOVA using the SAS software statistical package (Version 9.1, SAS Institute, Cary, NC). All data were reported as mean ± standard error of the mean (SEM). All data were determined to be parametric prior to performing Duncan's multiple range test to determine significant differences between lean and obese groups ($p<0.05$).

7.3 Results

7.3.1 Body Weight and Spleen Characteristics

DIO: Compared to lean rats, high fat-fed (HFF) rats had greater body (Table 7.3) and spleen weights and total number of splenocytes ($P<0.05$). However, when adjusted for body weight (bwt) spleen weight did not differ between lean and HFF rats. The concentration of splenocytes per gram spleen also did not differ between lean and HFF rats.

***fa/fa* Zucker Rat:** Obese Zucker rats had higher body and spleen weights and a lower spleen weight per gram (g) body weight, a lower number of total splenocytes and a lower number of splenocytes per g spleen weight than lean rats ($P<0.05$, Table 7.3).

JCR:LA-*cp* Rats: Obese JCR:LA-*cp* rats had a higher final bodyweight, and spleen weight than lean rats (Table 7.3, $P<0.05$). When adjusted for body weight, spleens of obese JCR:LA-*cp* rats weighed less than lean rats, but the concentration of splenocytes per gram of spleen did not differ between lean and obese rats ($P<0.05$).

Table 7.3. Characteristics of lean rats and obese high-fat fed, *fa/fa* Zucker and JCR:LA-*cp* rats.

	DIO		Zucker		JCR:LA-*cp*	
	Lean (n=8)	Obese (n=8)	Lean (n=10)	Obese (*fa/fa*) (n=10)	Lean (n=5)	Obese (*cp/cp*) (n=10)
Age (wk)	12		16		17	
Body wt (mg)	442 ± 9	523 ± 10*	328 ± 5	543 ± 13*	378 ± 4	592 ± 5*
% Increase in Body wt (%)	18		66		57	
Spleen wt (mg)	1433 ± 61	1673 ± 9*	580 ± 10	730 ± 50*	856 ± 23	1165 ± 40*
Spleen wt per g body wt (mg/g)	3.2 ± 0.1	3.2 ± 0.1	1.8 ± 0.0	1.3 ± 0.1*	2.3 ± 0.1	2.0 ± 0.1*
# of splenocytes per mg spleen ($x10^5$)	2.2 ± 0.1	2.4 ± 0.1	3.9 ± 0.3	2.8 ± 0.4*	5.3 ± 0.5	5.6 ± 0.4

Data represent mean ± SEM; n=8/group for DIO and JCR:LA-*cp* and n=10/group for Zucker rats.
* indicates statistically significant difference between lean and obese rats of same strain ($P<0.05$).

7.3.2 Splenocyte Phenotypes

DIO Rats: There were no significant differences in any of the phenotypes measured (expressed as % of total isolated cells), including $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD25^+$, $CD8^+CD25^+$, $CD11b/c^+$, $OX6^+CD80^+$, $OX12^+$, $OX6^+CD11b/c^+$, and $OX6^+CD80^+$ cells between HFF rats and lean controls (Table 7.4).

***fa/fa* Zucker Rat:** Obese Zucker rats had a lower proportion of $CD3^+$, $CD3^+CD4^+$ and $CD8^+CD25^+$ cells and had a higher proportion of $CD11b/c^+$ ($P<0.05$) and $OX6^+CD86^+$ cells in the spleen. There was no difference in the proportion of $CD3^+CD8^+$, $CD4^+CD25^+$, $CD4^+CD28^+$, $CD8^+CD28^+$, $OX12^+$, $OX6^+CD11b/c^+$, $OX6^+OX62^+$ and $OX6^+CD80^+$ cells between lean and obese Zucker rats ($p<0.05$).

JCR:LA-*cp*: Obese JCR:LA-*cp* rats had a higher proportion of $CD3^+CD4^+$ and $CD11b/c^+OX6^-$, but a lower proportion of $CD4^+CD25^+$, $CD11b/c^+OX6^+$, and $OX12^+$ (B-cells) splenocytes compared to lean rats. There was no difference in the proportion of $CD3^+$, $CD3^+CD4^+$, $CD8^+$, $CD3^+CD8^+$, $CD8^+CD25^+$, $CD11b/c^+$ and $OX6^+$ cells between lean and obese JCR:LA-*cp* rats ($p<0.05$).

Table 7.4. Splenocyte phenotypes of lean and obese DIO, Zucker and JCR:LA-*cp* rats

	DIO		Zucker		JCR:LA-*cp*	
	Lean (n=8)	Obese (n=8)	Lean (n=10)	Obese (*fa/fa*) (n=10)	Lean (n=5)	Obese (*cp/cp*) (n=10)
T-cells ($CD3^+$)	31 ± 1	31 ± 2	49 ± 2	42 ± 1*	46 ± 1	44 ± 1
Th cells ($CD3^+CD4^+$)	20 ± 1	22 ± 0	27 ± 2	23 ± 1 *	33 ± 0	33 ± 1
CTL ($CD3^+CD8^+$)	11 ± 1	8.9 ± 0.7	20 ± 2	18 ± 1	13 ± 1	13 ± 2
Th cells expressing IL-2 receptor ($CD4^+CD25^+$)	2.1 ± 0.1	2.2 ± 0.1	6.3 ± 1.0	6.1 ± 0.6	8.8 ± 0.5	5.9 ± 0.8*
CTL expressing IL-2 receptor ($CD8^+CD25^+$)	1.2 ± 0.1	1.0 ± 0.1	4 ± 0.5	3 ± 0.5*	3.6 ± 0.5	2.8 ± 0.5
Macrophages/ Monocytes ($CD11b/c^+$)	12 ± 1	15 ± 2	18 ± 1	21 ± 2*	12 ± 0.6	12 ± 1
Macrophages expressing MHC II molecule ($CD11b/c^+OX6^+$)	2.6 ± 0.3	2.3 ± 0.3	9 ± 0.9	9 ± 1.3	11 ± 0	6.7 ± 0.9*
B-cells ($OX12^+$)	53 ± 2	52 ± 2	35 ± 2	34 ± 2	42 ± 2	37 ± 1*

Data represent mean ± SEM. Values are a proportion of the total gated cells as determined by immunofluoresence. * indicates statistical difference ($p<0.05$) between lean and obese rats of same strain. Abbreviations: Th, T-helper cells; CTL, cytotoxic T-cells; MHC, major histocompatibility complex.

7.3.3 Cytokine Production

DIO Rat: Splenocytes of rats fed the high-fat diet produced more ConA-stimulated IFN-γ ($P<0.05$, Fig. 7.1) and less LPS-stimulated IL-10 compared to lean chow-fed rats ($P<0.05$, Table 7.5). There was no significant difference in production of ConA-stimulated TNF-α, IL-1β, IL-2, IL-4 and IL-10 and high-fat fed rats produced similar amounts of LPS-stimulated IFN-γ, TNF-α, IL-1β, IL-4 and IL-6.

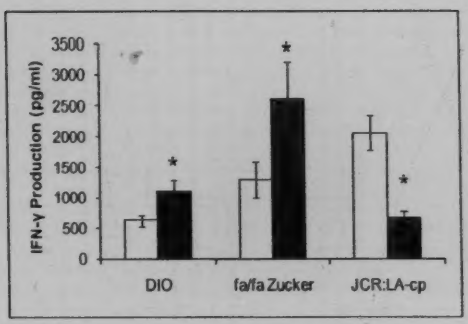

Figure 7.1. Splenocyte ConA-stimulated IFN-γ production from lean and obese rats. * Indicates statistical significance ($P<0.05$) between lean and obese rats of the same strain. ☐ lean; ■ obese.

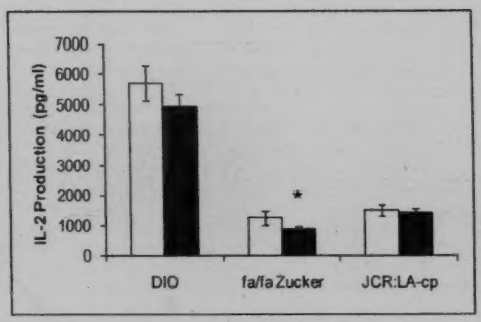

Figure 7.2. Splenocyte ConA-stimulated IL-2 production from lean and obese rats. * Indicates statistical significance ($P<0.05$) between lean and obese rats of the same strain. ☐lean; ■ obese.

Zucker Rat: Splenocytes from *fa/fa* Zucker rats produced more TNF-α, IL-1β and IL-6 than lean rats following LPS or ConA stimulation ($P<0.05$, Table 7.5 and Fig. 7.3). IFN-γ production in ConA stimulated splenocytes was higher in obese rats ($P<0.05$, Fig.7.1), but lower when stimulated with LPS ($P<0.05$, Table 7.5). Splenocytes from obese Zucker rats produced less ConA stimulated IL-2 than lean rats ($P<0.05$, Fig.7.2). LPS or ConA-stimulated IL-4 and IL-10 production did not differ between lean and obese Zucker rats (Table 7.5).

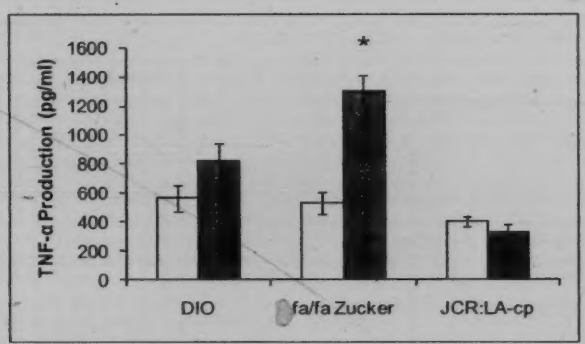

Figure 7.3. Splenocyte LPS-stimulated TNF-α production from lean and obese rats. * Indicates statistical significance ($P<0.05$) between lean and obese rats of the same strain. ☐ lean; ■ obese.

JCR:LA-*cp* Rat: Splenocytes of obese JCR:LA-*cp* rats produced less mitogen-stimulated IFN-γ (ConA and LPS), less LPS-stimulated IL-1β, and less ConA-stimulated IL-10 compared to lean rats ($P<0.05$, Table 7.5 and Fig 7.1). LPS-stimulated TNF-α, IL-2, IL-6 or IL-10 or ConA-stimulated IL-2 or IL-4 production did not differ between lean and obese rats ($P>0.05$, Table 7.5 and Fig.7.2).

Table 7.5. Splenocyte mitogen-stimulated cytokine production from lean and obese DIO, Zucker and JCR:LA-*cp* rats

		DIO		Zucker		JCR:LA-*cp*	
		Lean (n=8)	Obese (n=8)	Lean (n=10)	Obese (*fa/fa*) (n=10)	Lean (n=5)	Obese (*cp/cp*) (n=10)
		(pg/ml)					
ConA	TNF-α	167 ± 34	289 ± 99	870 ± 202	1160 ± 257	76 ± 24	74 ± 10
	IL-4	0.2 ± 0.03	0.2 ± 0.03	24 ± 4	16 ± 2	42 ± 10	82 ± 17
	IL-10	1760 ± 221	1805 ± 241	545 ± 120	543 ± 119	116 ± 7	50 ± 5*
LPS	IFN-γ	159 ± 62	217 ± 43	201 ± 55	106 ± 23	670 ± 263	134 ± 71
	IL-1β	167± 19	186 ± 15	378 ± 59	652 ± 101*	342 ± 33	254 ± 39*
	IL-6	621 ± 58	648 ± 25	3342 ± 301	4184 ± 320*	807 ± 97	595 ± 107
	IL-10	4250 ± 355	3289 ± 182*	846 ± 91	1066 ± 94	112 ± 8	89 ± 10

Data represent mean ± SEM. * indicates statistical difference ($p<0.05$) between lean and obese rats of same strain. Cytokine concentrations cannot be compared among groups (strains) due to differences in culture conditions and the commercial ELISA kits used.

7.4 Discussion

7.4.1 Immune Function in Human Obesity

Several research groups have identified impairments in immune function in the overweight population (refer to Chapter 1, section 1.3. for a comprehensive review). Although there is a general consensus that immunity is impaired, specific impairments and the underlying mechanisms have not been delineated. Due to the difficulty in controlling for the many factors that influence immune function when comparing obese and lean individuals, animal models have been employed to study the immune response in obese states. Several different animal models have been studied and there is currently no consensus regarding the appropriate animal model to use to represent human obesity. There has been no comprehensive comparison of the main animal models of obesity.

While we provide invaluable information with our comparisons for future study design, there are a few inherent limitations. The diets used among the rodent models differed, including the level and composition of dietary fat and the length of feeding. As such, we are limited in our ability to make direct comparisons among the models. However, for the JCR:LA-*cp* and the Zucker rodent the same diet was fed to lean and obese rats, which excludes the contribution of individual nutrients to differences in immunity. While we attempted to control for the composition of the high fat diet, the total quantity of fat was obviously much greater in the high-fat fed rats compared to lean, chow-fed rats. We were careful to exclude EPA, DHA and CLA from all three diets as these are the only fatty acids reported (at this point) to modulate immunity in obesity. Accepting these limitations, the following sections provide the first comprehensive comparative study of the models used to examine immunity in the obese state.

7.4.2 Characteristics and Distribution of Immune Cells

T-cells: It is likely that the lower proportion and concentration of T-helper cells in the spleen of *fa/fa* Zucker rats contributed to the reduced ability to produce IL-2. Several researchers have investigated the types and distribution of T-cells in human obesity, although reports are conflicting. While Tanaka *et al* (2001) (23) reported a lower percentage of $CD3^+$ and $CD4^+$ cells in the blood, this does not appear to completely explain the lower proliferative response of isolated T-cells they report. It is likely that the reduced proliferative response of T-lymphocytes was due to a defect in cellular function rather than a reduced proportion of cells in the assay that could respond. In contrast to Tanaka's report, others have reported an increased percentage or concentration of T-cells, including $CD4^+$ cells (24-27), which is consistent with the higher proportion of CD4+ splenocytes in obese JCR:LA-*cp* rats. It has been more consistently reported that the percentage or concentration of $CD8^+$ T-cells is lower in the blood of obese individuals (23, 25, 27). However, the proportion of splenocytes that were $CD8^+$ did not differ between lean and obese rats in any of the models studied.

B-cells: The proportion of B-lymphocytes present in the spleen did not differ between lean and DIO rats or lean and *fa/fa* Zucker rats. However, JCR:LA-*cp* rats had a lower proportion and hence a lower concentration of B-cells in the spleen. A few researchers have examined the concentration of B-cells in the blood of overweight

humans and the results are inconsistent. Two separate research groups reported that the concentration of B-cells in the blood were unaffected by obesity (23, 28); however, one report suggests that the concentration of B-cells was slightly higher (24). Thus, based on the current evidence *fa/fa* Zucker rats and DIO rats may have a distribution of B-cells more consistent with that observed in overweight individuals.

Macrophages/Monocytes: Similar to other cell types examined, the studies reporting the distribution of macrophages and monocytes in the blood of obese humans is limited. Comparable to the *fa/fa* Zucker rat splenocytes, it has been reported that overweight individuals have a higher concentration of circulating macrophages/monocytes (24, 26). However, one group has reported the there is no difference in the percentage of blood macrophages/monocytes (27). The increased proportion of these innate immune cells in *fa/fa* Zucker rats may partly account for the higher production of LPS-stimulated IL-1β, IL-6 and TNF-α. Similarly, it is possible that higher circulating levels of inflammatory mediators in obese individuals may be due to higher blood concentrations of monocytes. The lower concentration and function of T-cells could also have contributed to the higher inflammation in both humans and the *fa/fa* Zucker rat. In summary, human studies reporting immune cell phenotypes are conflicting, making it difficult to conclude which rodent model better represents human blood phenotypes.

7.4.3 T-cell Function

T-cells represent the largest percentage of lymphocytes in the blood and numerous lymph organs and they have a major role in cell-mediated and humoral immunity (29). Despite their importance, there have been few investigations into the impact of human obesity on T-lymphocyte responses. Tanaka *et al* (1993 and 2001) (23, 30) and Nieman *et al* (1999) (24) reported that the *ex vivo* proliferative response of whole blood or isolated blood T-lymphocytes to ConA and/or PHA was lower in obese humans compared to age-matched lean control subjects. We assessed T-cell function by the ability of splenocytes to produce IL-2 in response to a T-cell mitogen, ConA. Of the three models studied, only the *fa/fa* Zucker rat had an impaired ability to produce the T-cell proliferative cytokine, IL-2; while the HFF or obese JCR:LA-*cp* rat did not differ from lean rats. The contribution of leptin to T-cell function has recently been identified as critical. The leptin

receptor is expressed on T-lymphocytes and reports suggest that leptin is crucial for the optimal proliferative response of $CD4^+$ T-lymphocytes (31), prevention of T-lymphocyte apoptosis (32) and secretion of IL-2 (33). From this, it would be expected that a defective leptin receptor would result in lower mitogen-stimulated IL-2 production, as was seen in the *fa/fa* Zucker rat. Furthermore, it is also possible that the leptin resistance present in obese humans could contribute to the impaired ability to proliferate or produce IL-2 in response to a T-cell mitogen. In contrast to this hypothesis, obese JCR:LA-*cp* had normal ConA-stimulated IL-2 production, suggesting that leptin-mediated signalling may not be necessary for adequate IL-2 production in this model. Thus, an obese animal model with leptin related defects may not limit its use in studying T cell function.

Several important cytokines are involved in the mediation and regulation of T-cell responses; however, few studies have examined the ability of immune cells to produce these cytokines in obese individuals. The production of IFN-γ by isolated peripheral blood mononuclear cells in response to a T-cell mitogen was reported to be lower in morbidly obese patients (34). Similarly, obese JCR:LA-*cp* rats produced less ConA-stimulated IFN-γ, suggesting that this response may more closely represents that reported for morbid obesity. In contrast, it has been reported that obese subjects had a higher proportion of stimulated IFN-γ^+ $CD4^+$ T-cells (28) and similarly, higher ConA-stimulated IFN-γ was observed in DIO and *fa/fa* Zucker rats. This suggests that the stimulated cytokine responses of JCR:LA-*cp* rats may better represent those of morbidly obese; whereas, DIO and *fa/fa* Zucker rats have a greater Th1 responses, which may more suitably characterize this aspect of immunity in less severely obese humans.

7.4.4 Inflammatory Cytokines

Although there is strong evidence that obese individuals have higher circulating markers of inflammation, the stimulated response of innate immune cells has been poorly studied. Tanaka *et al* (2001) (23) was the only group to report that monocytes isolated from obese humans produced more TNF-α in response to *in vitro* LPS-stimulation. Similarly, splenocytes of *fa/fa* Zucker rats produced more LPS-stimulated TNF- α, IL-1β and IL-6. Whereas, the production of TNF-α or IL-6 did not differ between lean and obese DIO rats or lean and obese JCR:LA-*cp* rats. Moreover, splenocytes of obese JCR:LA-*cp* rats produced less LPS-stimulated IL-1β. Recently, Fontana *et al* (2007) (34)

reported that LPS-stimulated monocyte chemoattractant protein-1 (MCP-1) was lower in morbidly obese patients. This suggests that the inflammatory response of obese JCR:LA-*cp* rats may better represent human morbid obesity. Another possible explanation for the discrepancy between the two models is the activity or lack of activity of the leptin receptor. Although both models have genetic defects affecting the leptin receptor, the *fa/fa* Zucker rat has some residual receptor mediated signalling (35, 36), whereas the JCR:LA-*cp* rat does not. In recent years researchers have identified an integral role for leptin in the inflammatory response of immune cells (37). It is possible that the residual amount of leptin receptor activity is permissive to the production of inflammatory cytokines in stimulated cells of the *fa/fa* Zucker rat, while the complete lack of activity hinders production in the JCR:LA-*cp* rat.

7.4.5 Strengths and Limitations of Models

While animal models allow us to examine the effects of dietary intervention on tissues and organs and to a greater extent than human studies, there are limitations of using these rodents. One of the chief criticisms of using the obese JCR:LA-*cp* and *fa/fa* Zucker rats is the fact that the genetic mutation responsible for the obesity, is extremely rare in overweight/obese humans (3). Additionally, leptin has been recognized as a key mediator of both T-cell and inflammatory immune reactions (38). Leptin has been shown to modulate macrophage and dendritic cell function, inflammatory cytokine production, Th1 cytokine responses and the proliferation of $CD4^+$ T-cells (38). Clearly, leptin is an integral component of immune cell function and it has been argued that impairments in the genetic models are simply due to the lack of leptin activity and not obesity itself. However, leptin resistance is a consistent feature of obesity (39) and thus, likely contributes to the immune dysfunction present in human obesity. Furthermore, as we have identified similarities in immune function between the genetic models and human obesity and hence, the defective leptin receptor does not exclude the use of this model to address the effect of diet or other treatments on immune dysfunction.

The high fat fed DIO rodent model of obesity also has shortcomings. In the present comparative study, the DIO model was fed a high fat lard/corn oil diet, in which fat accounted for 59% of calories, and the lean control rats consumed chow (13.5% of calories as fat). Despite several claims that this model better represents human obesity,

there are a few obvious limitations, particularly for use in nutrition studies when dietary fat is the nutrient of interest. It is well documented that both the type and amount of fat in the diet can influence various components of immunity (40). High levels of dietary fat can suppress immune responses, T-cell function and antigen presentation (41-46) relative to low-fat diets. In addition to the high absolute amount of fat, there was a discrepancy between the chow (n-6):(n-3) PUFA ratio (6:1) and the HF diet (24:1); however, this was not due to differences in EPA or DHA and the absolute amount of (n-3) PUFA consumed would have been higher in obese rats due to a higher energy intake. Finally, the composition of the experimental diet, including the quantity of fat, used to induce obesity varied considerably from the composition of diets typically consumed by humans. For example, total fat accounts for approximately 33% of energy consumed in US diets (47) compared to approximately 60% of energy provided in the high fat rodent diets; whereas, the chow diet contained a very low level of fat ranging from 5-7% w/w fat or 13-15% of calories. Despite the high level of dietary fat, we observed few differences in the immune parameters measured, suggesting that the absolute quantity of fat had only a minor influence on immunity. Moreover, the lack of differences between the high fat and chow fed rats may also be explained by the smaller difference in body weight observed in the DIO model.

7.5 Conclusion

Overall, there are limitations to consider with any of the models used. Presently, it is difficult to distinguish the most suitable overall model for immunity in human obesity due to few studies and no studies that have compared the models. Our comparison suggests that both diet (likely dietary fat) and leptin sufficiency may influence aspects of immune responses. Careful consideration of the immune parameter of interest is required to determine which model is most suitable. Comparison of the DIO rat, the *fa/fa* Zucker rat and the obese JCR:LA-*cp* rat to the human literature indicates that the *fa/fa* Zucker rat may best represent the lower proliferative response of T-cells and the greater stimulated inflammatory cytokine production. However, the obese JCR:LA-*cp* rat may more adequately represent some of the immune changes reported in morbid obesity. Few differences in immune function were observed in the DIO model, suggesting that feeding a high fat diet (compared to a low fat diet of similar fat composition) has minimal impact

on immunity or that the degree of obesity (amount of adipose tissue) may significantly influence the immune response associated with obesity. Furthermore, due to the vast literature on the effect of different fatty acids on immune function, it is advised that future studies carefully consider this when designing protocols to study the effect of obesity on immune function.

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8 General summary and Discussion

8.1 Summary of Results

The overall objective of this research was to determine the effects of obesity and dietary polyunsaturated fatty acids on immune function.

1. **The first objective of this thesis research was to establish the effect of obesity on immunity. This objective was tested with the following hypotheses:**

a) Obese (*fa/fa*) Zucker rats will have impaired T-cell function and greater inflammatory responses compared to lean Zucker rats.

The results of Chapter 3 support this hypothesis. Obese (*fa/fa*) Zucker rats produced less ConA-stimulated IL-2, but more mitogen-stimulated IL-1β, TNF-α and IL-6 (inflammatory cytokines) and NO.

b) Obese JCR:LA-*cp* rats will have impaired T-cell and greater inflammatory cytokine responses compared to lean JCR:LA-*cp* rats.

This hypothesis is partly supported by results from Chapter 6, but is not supported by the results from Chapter 4. In Chapter 4, there was no difference in T-cell stimulated IL-2 production and there was lower stimulated inflammatory cytokine (IL-1β and IFN-γ) production by obese rats; whereas, the results presented in Chapter 6 found that production of an inflammatory cytokine was higher in obese rats. The results presented in Chapter 6 reported that IL-2 and IFN-γ production was higher in obese rats, indicating that the proliferative and Th1 response was greater in obese rats.

c) T-cell and inflammatory cytokine responses of MLN immune cells will be altered in obese JCR:LA-*cp* rats.

This hypothesis was supported by the results from Chapter 5. MLN cells produced more of an inflammatory cytokine (IL-1β) in response to mitogen stimulation.

Furthermore, T-cells produced more IL-4 suggestive of a Th2 biased response. Chapter 5 provided evidence that immune cells residing in visceral adipose tissue respond differently to mitogen stimulation, relative to peripheral immune cells (splenocytes), suggesting that proximity to adipose tissue influences immune cell responses.

d) High fat fed rats will have impaired T-cell and inflammatory cytokine responses.

The results presented in Chapter 7 partly supported this hypothesis. Feeding a high fat diet for six weeks had a minimal impact on immune cell phenotypes or inflammatory cytokine production. The production of IFN-γ, a Th1 cytokine, was greater and the production of the immunoregulatory cytokine, IL-10, was lower in high fat fed rats, indicating the T-cell function rather than the proportion of cells or their inflammatory response was affected by consuming a high fat diet.

2. The second objective was to determine the effect of changing the composition of dietary fat on immune dysfunction in obesity. It was hypothesized that:

e) Dietary CLA isomers will be incorporated into immune cell phospholipids and will improve T-cell and inflammatory cytokine production in obese (*fa/fa*) Zucker rats.

This hypothesis was supported by the results presented in Chapter 3. Feeding the c9t11 or t10c12 CLA isomer singly or in combination to *fa/fa* Zucker rats resulted in incorporation into splenocyte phospholipids, but to a lesser extent than lean rodents. Obese rats fed the c9t11 CLA isomer produced more IL-10 than those fed the control diet and the production of ConA-stimulated IL-2 by the CLA supplemented rodents was not significantly different from lean rats fed the same diet. Obese rats fed the t10c12 CLA isomer had lower LPS-stimulated IL-1β and TNF-α production.

f) Dietary long chain (n-3) PUFA will be incorporated into splenocyte membrane phospholipids and lipid rafts of obese JCR:LA-*cp* rats and will improve T-cell and inflammatory cytokine production in obese rats.

The results reported in Chapters 4 partly support this hypothesis. Obese rats fed fish oil had more long chain (n-3) PUFA and a lower (n-6):(n-3) PUFA ratio in splenocyte phospholipid membranes and lipid rafts. However, obese fish oil-fed rats produced lower levels of IL-1β and IFN-γ without affecting T-cell stimulated IL-2 production. Moreover, the higher level of EPA and DHA improved the inflammatory response to a greater extent than the lower level.

g) Dietary long chain (n-3) PUFA will be incorporated into MLN cell phospholipids and will improve stimulated T-cell and inflammatory cytokine production from MLN in obese JCR:LA-*cp* rats.

The results presented in Chapter 5 support this hypothesis. Obese rats fed fish oil had a great proportion of long chain (n-3) PUFA and a lower (n-6):(n-3) PUFA ratio in MLN cell membrane phospholipids. Both the low and high fish oil diet improved mitogen-stimulated production of the T-cell cytokines, IL-4, IL-10 and IFN-γ, as well as IL-1β. Data from this chapter indicated that both levels of EPA and DHA that were fed improved T-cell and inflammatory cytokine production, but these improvements were greater when the higher level of EPA and DHA were fed.

h) Dietary long chain (n-3) PUFA will be incorporated into splenocyte phosphatidylcholine and phosphatidylethanolamine and will modify T-cell and inflammatory cytokine production.

This hypothesis was supported by the results reported in Chapter 6. Feeding fish oil increased the relative proportion of long chain (n-3) PUFA and lowered the (n-6):(n-3) PUFA ratio in the major immune cell phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Both FO diets normalized ConA-stimulated IFN-γ production.

Obese rats fed the high fish oil diet produced more IL-2 in response to a polyclonal T-cell mitogen and FO normalized TNF-α production; whereas, the low fish oil diet did not affect production of this T-cell proliferative cytokine or TNF-α.

i) Dietary long chain (n-3) PUFA will modify T-cell stimulated IL-2 production by upregulating protein kinase C-theta (PKC-θ).

This hypothesis was not supported by the results presented in Chapter 6. Obese JCR:LA-*cp* rats fed the high fish oil diet had lowest levels of total protein kinase C-theta (PKC-θ) and phospho-PKC-θ in ConA-stimulated splenocytes. Feeding the low fish oil diet lowered phospho-PKC-θ, but not total PKC-θ, compared to feeding the control diet (no long chain n-3 PUFA).

3. To describe and compare the accepted rodent models of obesity and insulin resistance and determine which model best represents reports in human obesity. It is hypothesized that:

j) The immune responses in the high fat-fed obese rats will differ from that of *fa/fa* Zucker and JCR:LA-*cp* rats.

The results reported in Chapter 7 support this hypothesis. The diet-induced obese (DIO) rodent model had fewer immune abnormalities compared to the *fa/fa* Zucker or obese JCR:LA-*cp* rats. This may be attributable to the lower difference in body weight as compared to differences between lean and obese rats in the genetic models.

8.2 General Discussion

8.2.1 Gut-Associated Immunity

Our examination of MLN immune cell function is the first published report in rodents with obesity and insulin resistance. Our results indicate that this is a significant area for future research. MLN are an important tissue in the gut-associated immune system (GALT). GALT is the major site of antigen exposure in the body and the first contact of the immune system with dietary components. Any alterations in immune cell function in this site could impact whole body immune defense and oral tolerance. Unlike peripheral (splenocyte) immune cell function, stimulated MLN immune cells produced greater Th2 and inflammatory cytokine responses in obese JCR:LA-*cp* rats. Current evidence indicates that adipose tissue is an active endocrine organ that secretes an array of inflammatory and anti-inflammatory mediators (1). The accumulation of excess fat, an integral feature of obesity, coincides with a greater infiltration of macrophages (2) and T-cells (3), suggesting activation of both the innate and adaptive branches of immunity. Presently, it is unknown how the inflammatory environment of visceral adipose tissue contributes to the immune abnormalities of obesity. However, it is likely that the visceral adipose tissue, in which MLN reside, influences the function of these resident immune cells and vice versa. A very recent study by Kim and colleagues (4) suggested that factors emanating from adipose tissue, such as free fatty acids, reduced the viability of MLN immune cells in healthy, lean rodents. In consideration of this, it would be valuable to examine the interrelationship of adipose tissue and lymph node immune cell function in obesity. In order to study this and to build on the results from the experiments in the JCR:LA-*cp* rat, it would be pertinent to isolate the MLN cells from lean rodents and culture them with visceral adipose tissue from obese versus lean rodents. The concentration of cytokines in the media would be measured and functional assays could be performed on isolated T-cells, including stimulated proliferative and cytokine responses. Conversely, lymph nodes isolated from lean and obese rats could be cultured in lean adipose tissue to determine the effects of lymph nodes on adipose tissue inflammatory environment.

In addition, we demonstrated that dietary fish oil improved T-cell and inflammatory cytokine production in MLN immune cells. Emerging evidence indicates

that with respect to visceral adipose tissue, long chain (n-3) PUFA can reduce the infiltration of macrophages (5), reduce adiposity (6-9) and improve inflammation (5). Presently, it is unknown how increasing dietary long chain (n-3) PUFA affects the interaction of adipose tissue, infiltrated macrophages or T-cells and immune cells of resident lymph nodes in the obese state. To determine the effect of dietary EPA and DHA on adipose tissue and lymph node immune function similar experiments as outlined above could be conducted. Briefly, adipose tissue from obese JCR:LA-*cp* rats fed no, low or high EPA and DHA diets could be cultured with MLN immune cells from lean rats. In turn, the immune cells would be stimulated and the ability to produce cytokines would be determined (ELISA). In addition, the impact of EPA and DHA on visceral adipose tissue cellularity and the cytokines secreted would be assessed. Overall, these experiments would help us to determine the suitability of long chain (n-3) PUFA treatment on immune function and adiposity in obesity.

8.2.2 Fatty Acids: Proposed Mechanisms of Action

This research established that dietary polyunsaturated fatty acids can favorably modify immune function in obesity. The major dietary CLA isomers, c9t11 and t10c12, were incorporated into immune cell membranes, but to a lesser extent than lean rodents. To date, the underlying mechanisms involved in CLA-mediated immune modification have not been established, although a few have been proposed. CLA is a natural ligand and potent modulator of peroxisome proliferator activated receptor-gamma (PPAR-γ) (10) and this transcription factor is expressed in T-cells, B-cells and macrophages (11). Experiments conducted *in vitro* with the RAW macrophage cell line provided evidence that CLA can lower the production and/or expression of IL-1β, TNF-α and IL-6 (12). In the absence of functional PPAR-γ, the anti-inflammatory effects of CLA were repressed (12). This supports the hypothesis that CLA utilizes PPAR-γ to exert its anti-inflammatory effects. In recent years, the use of thiazolidinediones drugs in the treatment of insulin resistance, has revealed that these PPAR agonists also exert anti-inflammatory effects in obese subjects (13). To date, studies have focused on adipose tissue and little is known regarding the potential efficacy of PPAR-γ agonists on peripheral immune cell function in obesity. Our study indicated that the t10c12 CLA isomer can reduce production of inflammatory mediators after mitogen stimulation and I postulate that these

effects are mediated via activation of PPAR-γ in T cells and/or macrophages. In order to test this hypothesis and to build on previous experiments, I would use the *fa/fa* Zucker rat as the model and would isolate and separate macrophages and T-cells. Following stimulation of these cells, the expression (Western blot) and activity (transcription factor ELISA) of PPAR-γ would be determined.

As hypothesized, the fatty acid composition of whole membrane and lipid rafts were modified by dietary fish oil. Splenocytes of obese rats fed fish oil had more EPA and DHA in whole membrane phospholipids, including PE and PC. Furthermore, relative to the whole membrane, there was an even greater percentage increase in the incorporation of long chain (n-3) PUFA into splenocyte lipid rafts. This is the first study to demonstrate that dietary fish oil can modify the fatty acid composition of immune cell lipid rafts in obese rodents and exemplifies a potential mechanism. Our data suggested that at least the higher level of EPA and DHA can alter the expression of the MHC class II molecule. This could modify the ability of T-cells to adequately recognize antigen and mount a response. Previous studies have shown that EPA and DHA can downregulate the expression of MHC class II molecules on immune cells in healthy rodents (14, 15); moreover, this molecule has been shown to reside in lipid rafts (16-18). Hence, it would be pertinent to investigate whether EPA and/or DHA can displace MHC class II molecules from lipid rafts and how this would modify antigen presentation and subsequent T-cell responses in obesity. To continue with the observations made in the JCR:LA-*cp* rat, I would conduct a short-term feeding study (3 weeks) with similar experimental diets: no fish oil, low fish oil and high fish oil. Separation of antigen presenting cells (via magnetic bead sorting) and subsequent isolation of the lipid raft material would enable us to determine the effects of dietary EPA and DHA on the level of MHC class II molecules in the lipid raft fractions (via Western blot). Further experiments could be conducted *in vitro* to determine if EPA and DHA disrupt the formation of the immunological synapse, the interface between T-cells and antigen presenting cells (by confocal microscopy) and whether antigen presentation is disrupted.

Previous reports in cell culture systems and healthy rodents have established that long chain (n-3) PUFA can modify lipid raft phospholipid fatty acid composition, cholesterol content and protein composition of T-cells (19-24). We demonstrated that the

proliferative response of T-cells may be greater in obese rodents and even more so with high level of fish oil supplementation. While we attempted to establish that this response was mediated via PKC-θ, our results suggest that this is not the pathway that explains the IL-2 production that occurs in obese rats fed fish oil. In fact, feeding EPA and DHA significantly decreased both total (HFO only) and phosphorylated levels after stimulation. The impact of feeding fish oil on PKC-θ in immune cells is limited to one study in healthy mice (21). Fan *et al* (2004) (21) reported that dietary fish oil displaced PKC-θ from lipid rafts; but, unlike our report, this corresponded with lower IL-2 production (21).

One potential pathway by which EPA and DHA increased IL-2 production in the JCR:LA-*cp* rat may be via modification of calcium mediated signaling. EPA and DHA have been shown to modulate Ca^{+2} influx in T-cells (25) and this influx is required for IL-2 production with T-cell receptor stimulation (26). One group has reported that dietary fish oil lowered thapsigargin-stimulated Ca^{2+} influx in basal state T-cells and the authors postulated that this improved ConA-stimulated T-cell proliferation in obese rat pups (27). It has also been reported that EPA and DHA can modify calcium channels in other cell types such as neural and cardiac cells (28). However, it is unknown how EPA and DHA influence membrane-bound calcium channels, Ca^{+2} influx and/or activation of downstream transcription factors, such as nuclear factor of activated T-cells (NFAT), during T-cell activation in the obese state. In order to investigate this, a short-term feeding study using a diet composition similar to previous studies could be conducted in JCR:LA-*cp* rat. It would be necessary to isolate (via magnetic bead negative selection) and to stimulate $CD4^+$ T-helper cells to determine if EPA and DHA can modify calcium channel expression (flow cytometry), the intracellular concentration of calcium, and/or the activity of NFAT (transcription factor ELISA). Refer to Figure 8.1 for an overview of the potential mechanisms involved.

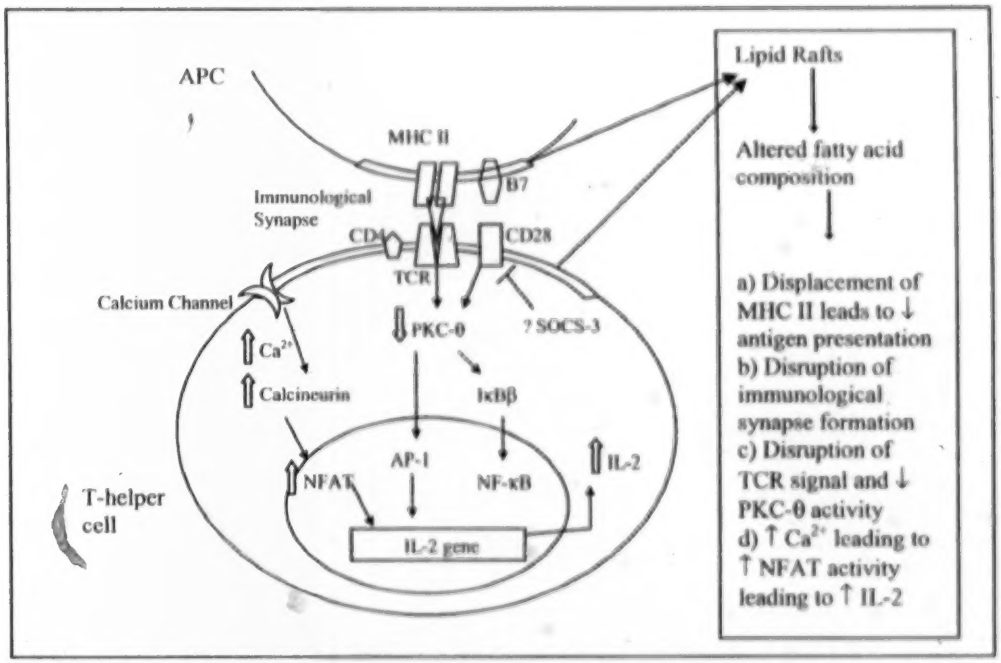

Figure 8.1. Schematic of the proposed mechanisms by which EPA/DHA could affect T-helper ($CD4^+$) cell stimulated IL-2 production in JCR:LA-*cp* rats.

8.2.3 Obesity and Immune Function: Beyond T-helper Cells

In agreement with the limited research conducted in overweight/obese humans, we demonstrated that T-cell function, as measured by mitogen-stimulated IL-2 production, was impaired in the *fa/fa* Zucker rat. This lower IL-2 response is likely attributed to $CD4^+$ or T-helper cells; however, it would be necessary to isolate these cells to exclude the influence of other cell types. Dendritic cells (DCs) and regulatory T-cells (T-regs) are of particular importance due to their role in establishing the type (Th1 vs. Th2 vs. Th17) and the robustness of T-cell responses. Both DCs and T-regs have garnered significant attention in recent years due to their role in antigen tolerance and inflammatory autoimmune diseases (29, 30). DCs are particularly important in antigen presentation because they are the only antigen presenting cell (APC) that can activate naïve T-helper cells (31). Recently, Macia *et al* (32) was the first, and so far only, to report that DC function is impaired in leptin deficient obese mice. More specifically, DCs from *ob/ob* mice were less able to stimulate T-cells in lean mice, which resulted in lower production of IL-4 and IL-10 (32). This study exemplifies that DCs may have a critical

role in influencing T-cell function in the obese state. In our experiments, the *fa/fa* Zucker rats had reduced T-cell activity; however, we did not assess DC function. To address this, it would be pertinent to isolate these cells from obese Zucker rats and determine if there are any functional or phenotype differences between lean and obese rats or if dietary long chain (n-3) PUFA modulate function. However, because DCs comprise a very small fraction of spleen immune cells, this would not be straight-forward to do. Building on the experiments conducted by Macia *et al* (32), we could increase our resource of DCs by collecting bone marrow cells and culturing under conditions that induce differentiation and maturation. In turn, we could assess the ability of DCs from obese rats to affect T-cell activation in obese or lean rats. In summary, DCs have a significant impact on the effector T-cell response and thus may contribute to the immune dysfunction present in the obese state.

T-regs, as their name implies, regulate the response of effector T-cells, including inducing antigen tolerance and facilitating the resolution of inflammation (29). As such, they may contribute to the T-cell and inflammatory dysfunction present in the obese state. One report in overweight subjects observed no difference in the proportion of blood T-regs (33); but, based on this experiment one can not eliminate the potential role of T-regs in mediating the immune dysfunction reported in the obese state. Researchers have shown that dysfunction of these cells may contribute to atherosclerosis (34), a condition which is more prevalent in obese individuals. In order to establish the effects of obesity on T-reg function I would employ the *fa/fa* Zucker rat and assess the proportion and concentration of this cell population in the spleen. Again, we are limited in our ability to assess function in this cell population due to the small number present in the lymph tissues. However, several methods have been developed which allow us to enrich this T-cell population (35), permitting more functional assays to be performed. The ability of T-regs from obese rats to control the development of Th1 and Th2 responses on immune cells from healthy, lean rats would initially be determined *ex vivo*. In addition, *in vivo* studies could be performed to determine the ability of T-regs from obese rats to influence the development of Th1 disease (i.e. arthritis) or Th2 disease (allergy). In summary, T-regs contribute significantly to the control of T-cell responses and govern antigen

tolerance highlighting the need to understand the role of these cells in the immune dysfunction of obesity.

The research reported in this thesis demonstrated that immune function is impaired in genetic and diet-induced models of obesity. It is the first scientific contribution exemplifying that the major CLA isomers, c9t11 and t10c12, can beneficially modify the immune response in the obese state. The research demonstrated that when long chain (n-3) PUFA are incorporated into a diet representative of human consumption, they can improve T-cell and inflammatory responses. Furthermore, this thesis presents the first comprehensive comparison of immune function of three major rodent models of obesity and provides a valuable resource for selection of rodent models for future study design.

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9 APPENDIX

Table 9.1. Summary of rodent studies reporting effect of obesity on immune function.

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
8-10 wk old C57BL/6J *ob/ob* and lean mice	Mice fed regular rat chow, ad libitum. Some mice were killed to harvest spleen and thymus for weight estimates. Remainder were injected interperitoneally with either sheep RBCs or EL-4 lymphoma tumour cells and killed several days later	Spleen, thymus	NK cell activity (^{51}Cr release), Ab-dependent cell-mediated cytotoxicity (spleen cells)	-↓spleen & thymus weight and ↓mononuclear cells -↓thy 1.2-positive lymphocytes (spleen) -↑ NK cell activity and Ab-dependent cell mediated cytotoxicity -↓Ab forming splenocytes, particularly IgG-producing cells -↓cytotoxic response of splenocytes isolated from injected mice, but no effect on T killer cells against alloantigens	(1)
6wk old lean and obese male Zucker rats	Rats given free access to rat chow for 18wks. Half of the rats were injected with *Candida albicans*	Peritoneal macrophages, PMNLs, kidney, lung, spleen, liver, heart, plasma	Phagocytosis assay, degree of infectivity	-↑susceptibility to systemic infection (*C. albicans*) -↑organ colonization of yeast -no diff in phagocytosis capacity -↓ability to kill phagocytosed yeast	(2)
6wk old lean and obese male Zucker rats	Lean and obese rats were equally distributed to the following treatments: sedentary control (ad libitum fed), sedentary calorie-restricted, exercise-trained and exercise-trained + calorie-restricted	Peritoneal macrophages	Phagocytosis and killing capacity	-no diff in phagocytosis capability -↓ability to kill phagocytosed yeast -↑candidacidal capacity with exercise training and caloric restriction (obese only)	(3)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
6 wk old male C57BL/6 *ob/ob*	*ob/ob* mice were fed ad libitum and isolated T-cells were treated with or without leptin	T-cells, naïve ($CD45RA^+$) or mature ($CD45RO^+$) and PBMC	Cytokines (mixed lymphocyte reaction(MLR)), proliferative response (MLR)	**Leptin (*in vitro*):** -↑ IFN-γ (all T-cells, $CD45RA^+$, $CD45RO^+$ or PBMC) -↑ IL-2 and proliferation (T-cells, but not mature T-cells) -↓ IL-4 production (T-cells, $CD45RO^+$ and PBMC)	(4)
8wk old female lean and obese Zucker rats	Rats fed non-purified diet until 12 months of age	Blood, spleen	Proliferative response (ConA or ConA+ Indomethacin, ^3H-thymidine), Prostaglandin E_2 production, ConA receptor expression, GLUT-1 protein levels, glucose uptake	-no sig difference in spleen weight per g body weight or in # of splenocytes per gram spleen -↑insulin and TG (plasma) -↓proliferative response to ConA, except at concentration of ConA need to induce max proliferation - ↓PGE2 production in ConA-stimulated splenocytes -↓glucose uptake into splenocytes (ConA) -↓expression of GLUT-1 after ConA stimulation -No sig difference in ConA receptor expression	(5)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
8wk lean and obese Zucker *fa/fa* rats	Rats fed water and lab chow ad libitum until 12mo of age. Half of the obese rats were assigned to an exercise group. Rats trained for 40wks	Blood/ plasma, spleen	^{3}H-Thymidine proliferative response (ConA), NK cell activity (% lysis of target cells measured by ^{51}-Cr release), glucose uptake (splenocytes)	-↓ConA-stimulated proliferative response -exercise improved proliferative response similar to lean rats -↓NK cell activity -exercise restored NK-cell activity -↓glucose uptake in ConA stimulated splenocytes), which was restored by exercise -↓GLUT-1 expression after ConA stimulation and improved, but not restored, by exercise	(6)
Male lean and obese Zucker rats (age not reported)	Tissue samples were collected from chow-fed rats from 5-38 weeks of age	Spleen, plasma, thymus	^{3}H-Thymidine blastogenic response (ConA, PHA, SEB), phenotypes	-T-lymphopenia (>8wks plasma, >11wks thymus and spleen) -↓CD4^{+} and CD8^{+} T-cells in obese rats -NK cells unaffected -↓blastogenic response at 11wks (T-cell mitogens), but not at 5wks	(7)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
In vitro study: *ob/ob* & *db/db* mice and lean controls; *In vivo* study: *ob/ob* mice and *fa/fa* rats	*In vivo* response to LPS injections as measured by cytokine production & *in vivo* phagocytic response as measured by clearance of Cr^{51},I^{125}-labelled E.Coli. *In vitro* response of peritoneal macrophages to LPS from *ob/ob* or *db/db* mice	*In vitro*: peritoneal macrophages; *In vivo*: serum, liver	Cytokine production, phagocytic function	-fewer phagocytically active peritoneal macrophages in both *ob/ob* and *db/db* mice (*in vitro*) -leptin ↑phagocytic responses of lean and *ob/ob* mice, but not *db/db* mice (*in vitro*) -*db/db* mice less capable of killing intracellular Candida (*in vitro*) -leptin ↑ IL-6, TNF-α, IL-12p70 & IL-12p40 (*in vitro*, peritoneal macrophages, LPS) -↓hepatic bacterial clearance and killing efficiency (*in vivo*) -↓TNF-α and IL6 production after *in vivo* LPS injections (*ob/ob* & *fa/fa* serum)	(8)
10 wk old C57BL/6 *ob/ob* or wildtype mice	*ob/ob* mice were randomized to: 1)Ad libitum fed; 2)injected with leptin; or 3) pair-fed with group 2 with PBS injections	Thymus, spleen	Phenotypes, histology, organ weights, cell counts and apoptosis	-↓ spleen weight -no differences in # of splenocytes or proportion of cell present -↓ thymocytes -↓ % of $CD4^+CD8^+$ (thymus) -↑ % of $CD4^+CD8$- and CD4-CD8- (thymus) -↑ thymocyte apoptosis Leptin administration: -spleen weight -↑ thymocytes -↑ % of $CD4^+CD8^+$ (thymus) -↓ (normalized) thymocyte apoptosis	(9)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
ob/ob mice and lean controls	Characterization of macrophage phenotype and function in *ob/ob* mice	Peritoneal macrophages	Phenotype, gene expression, PGE_2 production, IL-6 mRNA expression (LPS) oxidant and ATP production	-basal mRNA expression of *UCP*-2 was lower in obese mice, but expression did not change after LPS was added as it did with lean mice -↑H_2O_2 and O_2^- (basal and LPS) -↑ATP concentrations -↑COX2 expression (LPS) -↑ IL-6 (mRNA) and PGE_2 (basal & LPS) -↑DNA-binding capacity of C/EBP-β, an LPS-regulated transcription factor	(10)
4-5 wk old C57BL/6J *ob/ob* or wildtype mice	1. Mice were injected with ConA or Pseudomonas aeruginosa exotoxin A (PEA) to induce T-cell mediated hepatoxicity. 2. *ob/ob* mice were given leptin and then given ConA to induce liver toxicity.	Serum, liver, thymus	Cytokines (serum), hepatotoxicity (alanine amino-transferase levels), blood cell counts, phenotypes (thymus)	-↓ hepatotoxicity (ConA and PEA-induced) -↓ TNF-α and IFN-γ (ConA and PEA-induced) **Leptin Administration:** -restored hepatotoxicity to ConA -partly restored TNF-α -fully restored serum IFN-γ -restored the number of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and CD4-CD8- cells (reduced number in *ob/ob* mice) -normalized spleen weight (reduced in *ob/ob* mice) -restored serum levels of lymphocytes and monocytes	(11)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
4 wk old C57BL/6J mice fed high fat lard diet	Diet-induced obesity: mice were fed a high-fat diet [AIN 93 diet supplemented with lard (50% fat, 39% CHO, 11%pro)] or control mice were give AIN93 diet (10%fat, 74% CHO, 16%pro) for 13 weeks. Leptin was added *ex vivo* to splenocytes.	Spleen, adipocytes/tissue	Proliferative response (PHA or LPS, MTT assay), cytokine production (LPS or PHA),	-↑ TNF-α levels in visceral adipocytes -↑proliferative response to LPS, but no difference in response to PHA -↓IL-2 production (spl, PHA) -↑IL-4 and IFN-γ production (spl, PHA) -TNF-α (LPS) and IL-5 (PHA) production elevated in obese mice, but not significant **Leptin:** -↓IL-2 production (PHA, control mice) -↑IFN-γ production (PHA, ctl mice) -no effect on cytokine production in cells isolated from obese mice	(12)
5 wk old male Wistar rats fed low or high fat diet	Rats were fed AIN93 diet with or without high fat (lard) for 13 weeks	Spleen, blood	Cytokines, lymphocyte proliferative response (^{3}H-Thymidine incorporation), phagocytosis and respiratory burst	-↓ IL-2 production (ConA) -no change in IL-10, TNF-α or IFN-γ (ConA or LPS) -↓proliferative response (LPS and PHA, but not ConA) -↓$CD4^+$ T-cells -no effect on $CD8^+$ T-cells -↓oxidative burst capacity (blood monocytes) -no effect on phagocytic capacity (blood monocytes)	(13)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
4wk old c57BL/6J mice	Mice were fed one of 3 diets for 16wks: 1) High fat lard diet (50%w/w) 2) High fat soybean oil diet (50%w/w) 3) Ctl diet (fat 10% w/w) In approximately half of the mice, airway hypersensitivity was induced by ovalbumin (OVA) injection followed by aerosole ovalbumin exposure	Serum and spleen	Cytokine production and proliferative response (MTT assay), immunoglobulin levels	-↓ proliferative response obese rats fed either high fat diet (PHA) -↓proliferative response of Lard-fed mice (LPS) -no difference in proliferative responses of mice exposed to OVA Ag -no sig difference in IFN-γ production in OVA mice of either high fat diet (PHA) -↑IL-2 production in OVA mice in either high fat diet (*ex vivo* OVA stimulation of splenocytes) -↑proliferative response to OVA in OVA mice in lard high fat group -↑number of mast cells in OVA-soybean obese mice, but not OVA-lard mice -↓ OVA-specific IgG1 in OVA-lard obese mice -↓OVA-specific IgE in OVA-soybean obese mice -no sig diff in OVA-specific IgG2a, IgG or total non-specific IgG serum levels in either OVA-lard or OVA-soybean	(14)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
C57BL/6 *ob/ob* & lean mice and C57BLK *db/db* & lean mice	Half of the mice were immunized with BSA at day 0 and 7 and then arthritis was induced with an intra-articular injection of BSA on day 21	Synovial tissue, inguinal lymph nodes (ILN), serum, spleen	mRNA (synovial tissue), proliferative response (^{3}H-thymidine), cytokine production, Ig levels	-↓synovial inflammation (*ob/ob* mice) -↓synovial IL-1β and TNF-α in synovial tissue (*ob/ob* mice) -↓circulating anti-mBSA antibodies (*ob/ob*) -no sig diff in IgM, IgA or IgG (*ob/ob*) (*ob/ob*) - ↓anti-mBSA IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (*ob/ob*) -↓ILN proliferative response in *ob/ob* and *db/db* mice(mBSA stimulated) -↓IFN-γ production (*ob/ob*)	(15)
5 wk old male Wistar rats; diet-induced obesity	Rats were fed Rodent Toxicology diet rat chow or rat chow supplemented with pate, chips, chocolate, bacon and biscuits for 5 weeks; then rats were allocated to either ad libitum rat chow fed or 50% caloric restriction	Spleen, thymus, adipose tissue	Transcription factor mRNA and activity	-↑PPAR-γ1 mRNA expression in spleen -↓NF-κB binding capacity (spleen) -energy restriction did not change PPAR-γ1 mRNA expression (spleen) -energy restriction restored DNA-binding ability of NF-κB	(16)
6 mo old lean and obese female *fa/fa* Zucker rats;	Rats were obtained at 6mo of age and fed equal amts of low fat chow diet (duration unknown)	Serum	Inflammatory Markers	-no difference in MIP-1a -↑ VEGF, EGF-2	(17)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
C57BL/6 *ob/ob* & lean mice	Mice were used for: 1) *in vitro* LPS study; 2) *in vivo* IL-15 injections with/out *in vivo* LPS injections; 3) *in vivo* leptin injections	Kupfer cells (KCs), liver mononuclear cells, liver	Phenotypes, cytokine production(LPS)	**Kupfer cells, *ex vivo:*** -↑IL-12 production and mRNA expression (LPS) -↓ IL-15 mRNA expression (basal) -↑IL-15 mRNA expression (LPS) IL-15 Injections (liver mononuclear cells): -normalized $CD4^+$ natural killer T-cells **Leptin Injections (liver) :** -↑IL-15 mRNA expression before and after LPS injections -↑IL-12 and IL-10 mRNA expression post-LPS injections -no effect on IL-4 or IL-18 mRNA expression **Leptin Injections** (liver mononuclear cells): -↑$CD4^+$ natural killer T-cells, but levels were still below lean mice	(18)
Female *ob/ob* wildtype C57BL/6j mice	Mice were infected (intratracheal) with Gram-negative pneumonia (*Klebsiella pneumoniae*)	Alveolar macrophages, lung homogenate, blood	Cytokines (infected lungs), macrophage phagocytosis, leukotriene synthesis (lung macrophages), bacterial burden	-↑ bacteremia and mortality -↑ bacterial load (blood and lung) -no difference in leukocyte counts -↓ phagocytosis and restored by leptin -no difference in TNF-α, IL-12 or MIP-2 -↓ cysteinyl-leukotriene and LTB4 (infected macrophages) and leptin (*in vitro*) restored levels.	(19)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
4 wk old c57BL/6J mice female fed control high or low fat diet	Mice were fed one of 3 diets for 14wks: 1) High fat lard diet (50%w/w) 2) Ctl diet (fat 10% w/w) Mice were allotted to one of the following treatments: 1) 48 h starvation + PBS injection; 2) 48 h starvation + leptin injections; 3) ad libitum fed + PBS injection	Serum, spleen	Proliferation (MTT assay), splenocyte phenotypes and cytokines (anti-CD3 or PHA)	-↑ leptin (serum) **Ad libitum**: -no difference in proliferative response - no effect on IFN-γ (anti-CD3 or PHA) **Starvation + leptin**: -↓ proliferative response -no effect on IFN-γ (anti-CD3) -↑ IFN-γ (PHA) **Starvation + PBS**: -↓ proliferative response -no effect on IFN-γ (anti-CD3) -↑ IFN-γ (PHA) -no effect on IL-2 or IL-4 (PHA or anti-CD3)	(20)
8-12 wk old female C57BL/6J *ob/ob* and wildtype mice	Peritoneal polymorpho-nuclear neutrophils (PMN) were isolated and ability to phagocytise bacteria alone or coated with IgG, complement or IgG + complement was determined.	Peritoneal polymorpho-nuclear neutrophils (PMN)	Phagocytosis of bacteria	-↓ phagocytosis -*in vitro* and *in vivo* leptin administration restored phagocytic capacity of PMN -↓ phagocytosis when K. pneumoniae were opsonised with IgG and complement or complement alone -no difference in phagocytosis when K. pneumoniae was opsonised with IgG alone -↓ CD11b expression (required for binding to complement receptor)	(21)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
5 wk old male Wistar rats fed high fat or chow diet	Rats were fed 1 of 2 diets for 5wks: 1) rat chow: 18% pr, 76% CHO & 6% fat (% energy) 2) pate, chips, chocolate, bacon & biscuits with chow diet: 9% pro, 29% CHO & 62% fat (energy).	Spleen, thymus & adipose tissue	Proliferative response (ConA, PHA or LPS), phenotypes and transcription factor mRNA	-↓CD4+ -no effect on $CD8^+$ cells -↓LPS and PHA induced proliferative responses, but no effect in ConA stimulated cells - no effect on PPARγ-1 mRNA expression	(22)
5 wk old male Wistar rats fed high fat or chow diet	Rats were fed unspecified rat chow or rat chow supplemented with pate, chips, chocolate, bacon and biscuits for 5 weeks	Spleen, adipose tissue	Cytokines	- no change in glucose or insulin levels** -↓TNF-α and IL-6 mRNA levels (unstimulated, spleen)	(23)
8 wk old male C57BL/6 *ob/ob* and wildtype mice	Arthritis was induced by Zymosan A injection into knee joint and acute inflammation was determined at 1 & 3 d or chronic inflammation at 14 or 20 d	Knee joints, blood	Cytokines (mRNA expression), acute phase proteins, histology	-↑ cartilage damage and inflammatory infiltration -observed more severe arthritis in *ob/ob* synovial joints -no differences in cytokine mRNA in inflamed joint -↑ IL-6 (6hr post-injection) and serum amyloid A (1d and 3 d post-injection) -overall increase in acute phase response with delayed resolution.	(24)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
Female *db/db* & db/? mice and C57BL/6j *ob/ob* and ob/? mice	All mice infected with Listeria monocytogenes. Half of the *ob/ob* were treated with leptin and other half given PBS (control).	Spleen, liver	mRNA, bacterial growth and counts, immune cell phenotypes, histology	-↑mortality due to infection in both *ob/ob* and *db/db* mice - no diff in bacterial growth in spleen or liver - ↑bacterial number in liver for both *ob/ob*(48hr) and *db/db* (12hr) Liver Histology: -↑hydropic (adema) degeneration, abcess formation (24hr) & ↓mononuclear cell infiltration and ↓T-cell and MO present in both *db/db* and *ob/ob* mice. -↓neutrophils infiltrated in liver of *db/db* mice Liver Chemokine mRNA expression: *Db/db*: -↓MCP-1 (42hr post) and KC (24 & 42hr post), but no diff in MIP-2 *Ob/ob*: -↓MCP-1 (48hr), but no diff in KC or MIP-2 **Leptin** (in *ob/ob* mice vs *ob/ob* PS): -↓bacterial cell growth (liver), fatty & hydropic degeneration (liver), number of abscesses (liver) -improved MCP-1 levels similar to lean mice -KC & MIP-2 mRNA expression unaffected	(25)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
5wk old Sprague-Dawley rats fed high fat or chow diet	Rats were fed control (chow) or high fat diet (35% energy, meat & pastry pies, pasta, cake and chow) for 2 or 10 wks	Blood, plasma, alveolar macrophages	Cytokine (LPS), leptin, NPY	-TNF-α production (LPS-stimulated) did not differ between either groups fed high fat diets and the control group -no difference in LPS-stimulated production of alveolar macrophages; high-fat rats tended to have higher baseline levels of TNF-α production and therefore lower % changes after stimulation -↑NPY after 2wks of high fat diet, but not after 10wks	(26)
6-8 wk old *ob/ob* or wildtype C57BL/6 mice	Mice were intranassally infected with live mycobacterium tuberculosis for 0, 2, 5 or 10 wk	Spleen, lung, foot pad	Cytokines (spleen, tuberculin protein), phenotypes (lung), lung histology, lung bacterial load, DTH (foot pad)	-↑ lung bacterial load at 5 and 10wks -no difference in survival -↓ lymphocyte and ↑ PMN lung infiltration (histology) -↑ % $CD4^+$ (10wk) -↓ %$CD4^+CD69^+$ and $CD8^+CD69^+$ (2 & 5wk) -↓ % $CD8^+$ (10wk) -↓ IFN-γ and no difference in TNF, IL-4, IL-6 or IL-10 (lung, all time pt) - ↓ IFN-γ (2 & 5wk) and no difference in *ex vivo* IL-4 production -↓ DTH response; ↓ foot pad thickness and inflammatory infiltrate -leptin restored IFN-γ response	(27)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
6-8 wk old male C57BL-6 mice fed high fat or chow diet	Mice were fed *ad libitum:* 1) high-fat (59% energy) 2) high-sucrose (48%) 3) high-fat/high-sucrose diets (35%/40%) 4) Chow (12%) for 4-12 wk -some mice were injected intraperitoneally with LPS to induce liver injury.	Serum, liver, hepatic mononuclear cells, spleen	Cell phenotypes and intracellular cytokines, serum IFN-γ and alanine amino-transferase, histology, apoptosis (annexin-V+ and 7-AAD-)	-↓ hepatic NKT cells (all diets) -no difference in spleen NKT cells -↑ IFN-γ^+ and TNF-α^+ mononuclear, T- or NKT cells (high-fat) -↑ IFN-γ (serum, high-fat) -↑ alanine aminotransferase (serum, LPS) -↑ inflammation and necrosis (liver, LPS) -↑ IL-12 (hepatic mononuclear cells) -↑ NKT cell apoptosis	(28)
C57BL/6 *ob/ob* and wildtype mice	Leptin was administered *ob/ob* mice to determine effects on thymopoesis	Thymus	Thymocyte weight, count and phenotypes	Adiministration of leptin to *ob/ob* mice: -↓ thymus weight -↑ # of thymocytes -↑ % $CD4^+CD8^+$ and % $CD4^+CD8^+$/CD4-CD8- -↓ % CD4-CD8- -↑ # of $CD4^+CD8^+$	(29)
4 wk old C57BL/6J mice were fed a low or high fat diet	Mice were fed 1 of the following diets for 30 or 120 d: 1. low fat (10% energy) 2. high fat (45% energy)	Spleen	Nuclear transcription factor activity and Ob-Rb (leptin receptor) cell expression	-STAT-3 signalling did not change with leptin stimulation in obese rats (vs. basal); however, STAT-3 signalling ↑in lean rats in response to leptin stimulation -no difference in Ob-Rb expression (lean vs. obese)	(30)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
4wk old C57BL/6 mice fed low-fat (lean) or high-fat (obese)	Mice were fed ad libitum 1 of 2 diets for 20 wk: 1. high fat diet (50% fat, 40% CHO, 11% protein, as energy) 2. low fat diet (10% fat, 74%CHO, 16% protein as energy) Half of the mice were administered OVA in their water for 3 days (orally sensitized, OS). All mice were given an IP injection of OVA day3 and 7 days later another OVA injection was given	Serum, spleen	Cytokine, Ab titres (OVA-stimulated)	-↓IgG1 in orally sensitive obese mice compared to lean OS. -↓OVA-specific IgA (both OS & immune) -↓OVA-specific IgM (OS) -↓IL-2 (ob immune vs lean and ob immune vs OS) -no effect on IL-4 production -↓IL-2 & IL-10 in OS lean mice vs immune lean mice	(31)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
C57BL/6J, C57BL/6J-ob and BALB/c (for MLR only) female adult mice	Keyhole limpet hemocyanin (KLH) immunization given to 5 *ob/ob* and 5 control mice	Spleen, bone-marrow derived DCs (BM-dDCs)	Proliferative response (^{3}H-thymidine, ConA and KLH), phenotypes, Langerhans cell (LC) migration, DC activation (LPS), cytokine, leptin and Ig, endocytosis, mixed lymphocyte reaction (MLR)	Spleen: -↓proliferative response to KLH (KLH immunized), but no difference with ConA stimulation -↑IFN-γ and IL-10 (not significant) -↓total IgG and IgG1 specific to KLH (immunized) -↑$CD11c^+/MHCII^+$ (naïve cells) BM-dDCs: -no difference in $CD11c^+$ (basal) -no difference in activation markers or cytokine expression after LPS treatment -no difference in endocytosis capacity MLR: -↓ability of BM-dDCs to stimulate T-cells -↓IL-10 and IL-4 in cultures with *ob/ob* DCs -↑TGF-β Migration Assay: -↑higher number of epidermal LCs -↑IL-4 and CCR7 mRNA expression in ear skin of *ob/ob* mice Leptin: -induced LC migration in *ob/ob* mice	(32)
12-16wk old lean or obese male Zucker rats		Plasma	Cytokine	-↑plasma TNF-α levels -↑protein expression of TNF-α in coronary arteries	(33)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
12 wk old male *ob/ob* and wildtype C57Bl/6 mice	Mice were infected intranasally with Gram positive Streptococcus pnemoniae or Gram negative Klebsiella pneumoniae	Lung homogenates, blood	Cytokines and chemokines (lung), bacterial burden	-↓TNF and IL-1β (S. pneumoniae) -↓ TNF, IL-6, MIP-2 (K. pneumoniae) -no difference in IL-10 -no difference in lung inflammation histological scores -no difference in lung or blood bacterial loads	(34)
6-8 wk old female DO11.10 α/β-TCR transgenic mice fed chow or high fat diet	Mice were fed chow or high-fat (36%w/w) diet for 11wk; mice were immunized with OVA to examine naïve T-cell responses	Peritoneal macrophages, spleen, inguinal lymph nodes, T-cell isolated from spleen	Proliferation (^{3}H-Thymidine, OVA or ConA), cytokine production (OVA or ConA), phenotypes, NO production (LPS+IFN-γ)	-no difference in # of splenic mononuclear cells, % of B- or T-cells or MHC II$^+$ cells -↑ % dendritic cells -↑ NO production (peritoneal macrophages) -↑ % of macrophages in peritoneal cavity -no difference in T-cell proliferation (OVA) -↑IFN-γ/IL-4 ratio (OVA and ConA) OVA immunization: -no difference in immune cells present in spleen -↓ T-cell proliferation (OVA & ConA) -↓ Proliferation when OVA peptide presented by APC's of high-fat fed mice to T-cells of lean mice -↑ proliferation when OVA peptide presented by APC's of lean mice to T-cells of high-fat fed mice. -↓ MHC II expression - no difference in IFN-γ/IL-4 ratio	(35)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
C57BL/6 mice fed standard chow or high fat (age not reported)	Mice were fed standard chow or high fat (60% energy) diet for 16 wk. Infection was induced a) systemically (i.v. injection of *phyromonas gingivalis*) or b) orally (teeth exposed to *p gingivalis* for 10 d); peritoneal macrophages were also isolated and incubated with P. gingivalis and/or LPS	Serum, peritoneal macrophages, teeth/plaque	Bacterial titres, cytokines, transcription factor expression	-↑ P. gingivalis in plaque samples and ↓ clearance of bacteria -↓ TNF-α (serum, oral infection) -↓ TNF-α and IL-6 (1 & 2 h post-systemic infection) -↑ SAA (basal) -no difference in basal levels of TNF-α and IL-6 -↓ inflammatory cytokine (TNF-α, IL-1β, MIP, MCP-1, GM-CSF, IL-12, RANTES and IL-6) response to P. gingivalis (peritoneal macrophages, *in vitro*) -↓ TNF-α (peritoneal macrophages, LPS) -↑ inflammatory gene expression (NF-κB, IL1rl, Traf3, Rel, Tlr4) (Peritoneal macrophages, LPS) -↓ anti-inflammatory gene expression (raf6, NFkbia, Csf3, Icam 1, Ripk 1, Rela, Tnfaip3 and Traf5) (peritoneal macrophages, LPS)	(36)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
C57BL/6J mice fed either low fat/no sucrose (lean) or high fat/sucrose diets (obese) for 22wk	Mice were give 1 of the following diets for 22wk: 1. Low fat (5%w/w) - coconut and soybean oil. 2. High fat (36%w/w) - coconut and soybean oil. Mice were intranasally infected with Influenza virus A at 22 wks and samples were collected at 0 day (uninfected), 3 or 6 day post-infection (PI)	Lungs, serum, spleen	Viral titres(lung), NK cell cytotoxicity (lung & spleen), lung histopathology, cytokine mRNA (lung), insulin & glucose, leptin	-↑serum insulin (PI) -↑blood glucose at baseline -↑serum leptin at baseline -↓ at 3 d PI - ↑(6.6 fold) in mortality -↓viral titre at day 6 PI -↑lung pathology ($p<0.1$) -↓NK cell cytoxicity (spleen & lung) -↓proportion of NK cells (lung only) -↓IL-18 mRNA(required for NK cell activity) -no effect on IL-12 or MIP-1α mRNA expression - ↓IL-1β, IL-6 and TNF-α at day3 (PI, mRNA) - mRNA levels of IL-6 and TNF-α remained elevated at 6 d PI, while levels in lean rats were diminished - ↓IL-10 3 d PI and did not differ from lean at day6 PI -↓MCP-1 and RANTES	(37)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
6-8 wk old Female Balb/C and C57BL/6 mice were fed a high fat or low fat diet	Mice were fed one of 2 diets for 28d: 1. Low fat diet (10% of energy) 2. High fat diet (45% of energy) Half of the mice were sensitized topically (abdomen) with 1% 2,4,6-trinitrochloro-benzene (TNCB). TNCB was applied to the ears of all mice 7 days later. Immune cells of sensitized mice were transferred to naïve mice and TNCB was applied topically. Mice were sensitized to OVA and injected with peritoneal exudate cells that had been cultured with OVA 9 days later.	Ear, draining lymph nodes, spleen	Gross morphology (ear swelling), contact hypersensitivity (CHS), adoptive transfer, immuno-histochemistry, cytokines (OVA) and OVA-specific Ig (ELISA)	- ↓ ear swelling (CHS) -↓IFN-γ production (CHS, draining lymph node cells) & non-sig↓in IL-4 - no ear swelling in mice who received immune cells from obese mice (ear swelling when lean mice immune cells were transferred) -no difference in # of Langerhans cells (ear) -no effect on IFN-γ or OVA-specific IgG1 and IGG2a/2b in OVA-sensitized mice	(38)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
8-12wk old female C57BL/6j-*ob/ob* and C57BL/6j lean mice	Leptin was injected prior to and following injection of *Streptococcus pneumoniae* (pulmonary bacteria)	Lung, blood, broncho-alveolar lavage (BAL) fluid and alveolar macrophages	Cytokines (ELISA), leptin, phagocytosis, pulmonary bacterial load, PGE_2, and H_2O_2 production	-↓survival -↑ pulmonary bacterial load -↑ leucocytes in BAL fluid -↑ MIP-2, TNF-α, and PGE_2 (lung) -no difference in IL-6 -↓ phagocytosis of *S. pneumoniae* -↓ H_2O_2 production in response to *S. pneumoniae* (reduced bactericidal activity) Leptin administration to *ob/ob* mice: -↑ survival of *ob/ob* mice -↑ pulmonary bacterial clearance -↓ bacterial circulation in periphery -↓ MIP-2, TNF-α, and PGE_2 (lung) -↑ phagocytosis of *S. pneumoniae* -improves H_2O_2 production to *S. pneumoniae*	(39)

Species	Study Description	Tissue/ Organ	Outcome Measure	Major Findings	Reference
8 wk old male C57BL/6 mice fed chow (lean) or high fat diet (obese)	Mice fed high fat (45% energy) diet (lard/soybean oil) or chow (13% energy for 3 mo. Freshly isolated cells was used to determine phenotypes, histology and apoptosis. Immune cells from lean mice were also cultured in the presence of free fatty acids, MCP-1, H_2O_2 or chylomicrons.	Mesenteric lymph nodes (MLN) and isolated MLN immune cells	Phenotypes, histology, apoptosis (TUNEL assay & Annexin V expression), cell viability (MTT assay); and adipose tissue free fatty acids (FFA), ROS and lipid peroxidation	-↓ MLN weight -↓ lymphoid cells -no morphological changes to MLN -↑ apoptotic cells (TUNEL-positive) -$CD4^+$ and $CD8^+$ no difference (no overall percentage differences) **When adjusted for # of cells per g lymph node:** -↓ T-cells, $CD4^+$ and $CD8^+$, $CD4^+Foxp3^+$ (regulatory T-cells) and $CD4^+CD25^+$, neutrophils, dendritic cells and B-cells -↑$CD4^+CD62L^{low}$ and $CD4^+CD44^{high}$ $CD8^+CD62L^{low}$ and $CD8^+CD44^{high}$ (activated T-cells) -↑ $CD4^+$ or $CD8^+$ positive for Annexin V (apoptotic T-cells) **Cultured Lymphocytes (lean mice):** -↓ cell viability (FFA, chylomicrons & H_2O_2) -↑ apoptotic cells (FFA & H_2O_2) -MCP-1 no effect on cell viability Mesenteric adipose tissue factors: -↑ FFA, ROS and lipid peroxidation vs lean mice	(40)

Table 9.2. Summary of studies on immune function in human obesity

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
24 male and 170 female health care workers	Hepatitis B vaccines were administered to antibody negative subjects at t=0, 1mo & 6mo.	Serum	Ab levels following vaccination	- ↑BMI correlated with lack of detectable AB response to HepB vaccine	(41)
34 obese (BMI=38.4±2) and 35 lean (BMI=21.3±0.4) men and women matched for age and sex	Case-control study. Subjects provided one fasted blood sample	Blood, Macrophages, T-cells and B-cells were isolated	White blood cell counts, proliferative responses (^{3}H-thymidine, PHA, ConA and PWM)	-↓T-lymphocyte proliferative response (PHA & ConA) -↓ B-lymphocyte proliferate response (PWM) - no difference in total lymphocyte counts	(42)
427 pre-adolescents (47% male and 53 % female) vaccinated HepB at 0, 3 and 6 months	Cross-sectional study: 12 year old children were randomly selected from pre-adolescent population that received HepB vaccination	Blood	Anti-hepatitis B titres	-↓ Anti-Hepatitis B titres compared to non-obese children	(43)
116 overweight or obese (>25kg/m^2) 44.3±9.7yrs & 41 lean (<25kg/m^2) subjects 42.2 ±10.9yrs	Cross-sectional study	Blood, peripheral blood mononuclear cells	White blood cell counts, phenotype, NK cell cytotoxicity, proliferative response (^{3}H-Thymidine, ConA, PHA & PWM) & phagocytosis and oxidative burst	- ↑ total WBC count, including higher levels of neutrophils, monocytes, CD4$^+$ T cells, B cells. -no difference in NK cells and CD8$^+$ T cells. -↓proliferative response to PWM, ConA & PHA (whole blood) -↑ phagocytosis and oxidative burst (neutrophils & granulocytes) -no difference in NK cell activity	(44)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
3512 children aged 8-16	Cross-sectional study NHANES III. Fasted blood samples collected	Fasted Blood	White blood cell counts and serum hsCRP	-higher prevalence of ↑CRP levels in overweight boys and girls -↑white blood cell count	(45)
22 obese women and 12 obese men aged 18±68 years and 38 lean women and 12 lean men, 19±52 years of age	Fasted blood samples were collected from lean and obese adults at baseline. To determine the effect of weight reduction on immune function, 23 obese subjects underwent weight loss and subsequent blood draws and other measures were taken	Fasted blood/serum, isolated mononuclear cells, isolated T-cells	Cytokines (LPS), proliferative response (^{3}H-Thymidine), white blood cell counts	-↓ proliferative response of T-cells to ConA and PHA -↓$CD3^+$, $CD4^+$ and $CD8^+$ -↓$CD4^+CD45RO^+$ and TCRαβ - no difference in CD4/CD8, $CD4^+CD45RA^+$, $CD3^+CD25^+$, TCRγδ$^+$, $CD19^+$, $CD16^+CD57^+$, $CD16^+CD57^-$,CD16-$CD57^+$ -↑TNF-α, sTNFR1 & sTNFR2 (serum) -↑TNF-α (basal or LPS-stimulated mononuclear cells) Weight Reduction: -improved ConA and PHA stimulated proliferative responses -↑ $CD3^+$, $CD4^+$, $CD4^+CD45RO^+$, TCRαβ and $CD16^+CD57^+$ -↓LPS-stimulated TNF-α production -all other factors listed above were not significantly altered	(46)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
1653 subjects Age: 55-74 Healthy, IGT or Type 2 Diabetic subjects	Population survey in southern Germany	Serum, plasma	Cytokines, acute phase proteins	-↑IL-6 levels (serum, IGT & Type 2 subjects compared to healthy) -↑soluble IL-6 receptors -no change in TNF-α levels, TNF-α-R60 or TNF-α-R80 (serum) -↑CRP, SAA & fibrinogen (plasma, IGT & Type 2 subjects)	(47)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
Obese and healthy non-obese children aged 6-13	Obese children recruited from Service of Nutrition in Buenos Aires, Argentina. Simple salivary and blood sample taken from each subject	Saliva, serum	Igs & complement	-↓sIgA levels -no difference in serum IgA levels -↑C3c (complement protein)	(48)
Lean & obese normal glucose tolerant & obese impaired glucose tolerant subjects 20-50 years of age	Cross-sectional study of 30 obese subjects with IGT, 32 obese subjects with normal glucose tolerance and 42 lean healthy control	Plasma	HbA1c, plasma lipids, TNF-α, sTNFR1 and sTNFR2, C-reactive protein (CRP), and leptin, insulin sensitivity	-↑TNF-α in obese-IGT compared to control-NGT women -↑sTNFR1 & sTNFR2 in obese-IGT compared to both groups -↕sTNFR1 and sTNFR2 in obese-NGT compared to ctl -sex specific differences	(49)
25 morbidly obese, 24 gastric banding patients and 13 normal weight female (?) subjects	Blood samples were collected from all subjects; patients that received gastric banding (GB) had blood samples taken prior to and following surgery	Blood	Nutrient status, blood lipids, CBCs, IF, cytokines, Igs & inflammatory markers	-↑leukocytes, PMN (obese vs lean & GB vs lean) & lymphocytes (obese vs lean) -↑CD4 (O & GB vs lean) -↓CD8 (obese vs lean & GB vs lean) -↑CRP, orsomucoid, C3, C4 (both obese vs lean) -↓leukocytes after GB, comparable to lean levels -no difference in Igs	(50)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
21 obese and 21 age and sex-matched non-obese subjects	Blood samples and anthropometric measures were collected from all subjects.	Peripheral blood mononuclear cells	NK cell cytotoxicity assay (lactate dehydrogenase release)	-no difference in spontaneous NK cell activity or IL-2 stimulated activity -↓sensitivity of NK cells to glucocorticoid suppression	(51)
24 overwieght (32.9 yrs), 102 obese (A: 81 w/ BMI 30-40 & B: 21 w/ BMI >40.9), 28 lean women	Fasting serum and plasma and anthropometrics were collected from each subjec	Serum, plasma	Serum NO, TNF-α, s-TNF-R1 & 2, plasma lipids, glucose & insulin	-↑NO in overweight and obese subjects -↑TNF-α in overweight and obese subjects (individually & combined) -no differences in s-TNF-R1 & 2 -↑insulin in overwt and obese subjects -correlations between BMI and NO, TNF-α & insulin -correlation between % body fat & weight and NO, TNF-α & insulin	(52)
16 lean (36.9 yrs) and 16 obese (43.2 yrs) female subjects	Fasting plasma samples were taken from each subject;	Plasma, MNC	Fasting insulin, FFA & glucose, NF-κB binding activity, cytokines, acute phase proteins, transcription factor activity	-↑Insulin, HOMA-IR, FFA -↑NF-κB binding activity and ↓Iκ-Bβ (MNC) -↑mRNA expression of TNF-α, IL-6, MMP-9, MIF (MNC) -↑CRP, TNF-α, IL-6, MIF, MMP-9 (plasma)	(53)
71 adolescents (15- 16 yrs) 39 obese and 32 non-obese (Asian subjects BMI>25kg/ m^2 for males and >23kg/m^2 for females)	Lean or obese Asian adolescents; fasted blood samples taken from each subject.	Serum	Fasted serum cytokines, anthropometrics	-↑TNF-α, sTNF-R1 & sTNF-R2 -positive correlation between TNF-α, sTNF-R1 & sTNF-R2 and BMI - positive correlation between TNF-α, sTNF-R1 & sTNF-R2 and waist cicumference	(54)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
10 lean and 10 obese female subjects	Blood samples were collected from each subject *It should be noted that obese subjects had several co-morbidities including diabetes and coronary artery disease. Caution should be taken in interpretation	Blood	Immune cell phenotypes	-↑$CD3^+CD4^+$, $CD3^+CD8^+CD95^+$ -↓$CD3^+CD8^+$ - no differences in $CD3^+CD4^+CD95^+$, $CD14^+$, or the expression of CD62L, CD28, or CD16 on T-cells or monocytes	(55)
41 obese and 42 lean female subjects without history of asthma	Fasting blood samples and anthropometrics were taken from each subject to examine prevalence of atopy in obese women	Blood	Cytokines, IgE, leptin, IR, OGTT, body composition	-↑specific IgE, but not total IgE -↑leptin -IL-4 not detectable -↑fat mass -↑C-peptide, glucose, insulin and IR (fasting)	(56)
63 obese and 63 lean pre-pubertal childeran (Tanner Stage 1)	Blood samples and anthropometrics were obtained after an overnight fast	Blood	Pro-inflammatory mediators & leptin	-↑leptin, IL-1β, IL-6, TNF-α -↓IL-2	(57)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
Case-control Study: 6 obese and 8 normal weight women	Subjects under went an oral glucose tolerance test; blood samples were taken at prior to and immediately following OGTT	Plasma, mononuclear cells	CRP, TNF-α (fasting plasma or LPS-stimulated)	-↑CRP and TNF-α (fasting plasma) -↓LPS-stimulated TNF-α production in lean subjects, post-OGTT. Overweight subjects did not suppress TNF-α production in the face of hyperglycemia suggesting an association between inflammation and insulin resistance. Failure to suppress TNF-α production may drive insulin resistance	(58)
15 normal weight and 15 overweight children/adolescents (8-17 yrs)	-fasting blood samples were taken from each subject and DEXA scans were performed, VO2 max test performed	Serum	Plasma cytokine and immunoglobulin levels	-↑IL-6 plasma levels -↓IgG anti-tetanus titre, although still higher than the recommended threshold levels. -no sig diff in plasma TNF-α, IL-1β or IL-1ra -no sig diff in plasma IgM, A, IgG and 4 IgG subclasses	(59)
15 lean & 24 overweight children (male & female), aged 6-18yrs	-anthropometrics, physical fitness (peak aerobic power) and blood samples collected from each subject	Blood	CBC, IF	-↑total leukocytes, granulocytes, neutrophils, monocytes and total lymphocytes -↑% of $CD3^+$ and $CD4^+$ T-cells -aerobic fitness only minimally affects leukocyte counts	(60)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
43 obese and 43 non-obese children, aged 6-9yrs	-fasted blood samples and anthropometrics were collected from each subject	Blood	Cytokines, blood lipids, glucose, insulin, IR, vitamin status	-↑insulin, IR, TAGs -↓HDL-C -↑leptin, fibrinogen, CRP and PAI-1 -no difference in serum IL-6 levels -↑homocysteine levels in hyperinsulinemic obese children -positive correlation between homocysteine levels and IL-6 & CRP	(61)
9-10 obese men & women (aged 45-46) and 9-10 lean men & women (aged 35-37)	-serum was collected -PBMC were culture with or without leptin from a separate group of lean and obese subjects *Obese subjects had pre-existing co-morbidities including diabetes and were on various medications including hypoglycaemic agents, all which would confound immune outcome measures	Blood, polymorpho-nuclear cells (PBMC)	Cytokines, mRNA	-↑IL-1RA (serum) -↓TNF-α (serum, PBMC) -no difference in serum IL-6 levels and IL-1β levels were undetectable -↓IL-1β, TNF-α and IL-6 transcript levels (PBMC) -no difference in IL-1RA transcript levels (PBMC) **Leptin** -↑IL-6, IL-1β and TNF-α in both lean and obese subjects	(62)
50 obese (20 pre-pubertal/30 pubertal) and 20 lean (7 prepubertal/ 13 pubertal)	Gasted blood samples collected	Blood	Intracellular cytokines (PMAI-stimulated), blood lipids, hormones and immune cell phenotypes	-no difference in total T and B cells, Th cells suppressor cells and NK cells -no difference in IL-2 or IL-4-CD4 secreting cells (PMAI-stimulated) -↑IFN-γ secreting $CD4^+$ T-cells	(63)

Subject Description	Study Description	Blood Component	Outcome Measures	Major Findings	Reference
children				(PMAI-stimulated)	
378 overweight men and 493 overweight women (>65yrs)	Prospective study in aging population (InCHIANTI), cross-sectional data; subjects were separated according to central or global obesity and muscle strength	Serum and Plasma	Cytokine and CRP levels	-↑IL-6 (serum), sIL6-r (central obesity)	(64)
33 lean, 19 overweight & 27 obese children (6-14 yr)	Fasted blood samples from Swiss children.	Plasma	Cytokines, leptin and CRP levels	-↑ CRP (overweight & obese) -↑IL-6 (obese) -↑leptin (obese>overweight> lean) -TNF-α - no difference among groups	(65)
322 lean, overweight, obese or morbidly obese women (35 ± 8 yr)	HIV-uninfected women were enrolled in the Women's Interagency HIV Study as control subjects; some subjects were Heptatitis C positive	Blood	Phenotypes	-↑ $CD4^+$ and white blood cell count (overweight, obese & morbidly obese) -↑ $CD8^+$ count (morbidly obese)	(66)
79 male and 70 female subjects admitted to hospital	Subjects with bacteremia were recruited from hospital and relationship was BMI was determined	N/A	Morbidity and mortality associated with BMI	-↑ mortality due to bacteremia (including type 2 diabetics) -↑ median BMI among those that died	(67)

12 obese and 10 lean children	Obese subjects were recruited prior to enrolment in weight loss program; age and gender matched controls.	PBMC	Phenotypes and intracellular cytokine staining	-no difference in CRP -no difference in % of T-regulatory cells, dendritic cells, TNF-α and IL-12 positive monocytes -no difference in the % of $CD4^+$ cells positive for IL-2 or IFN-γ -↓ % of $CD4^+$ cells expressing IL-4 -↓ % of IFN-γ^+/IL-4^+ $CD4^+$ cells	(68)

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